Liposomal glucocorticoids:

pharmaceutical, preclinical and clinical aspects

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Liposomal glucocorticoids:

pharmaceutical, preclinical and clinical aspects

Liposomale glucocorticoïden:

farmaceutische, pre-klinische en klinische aspecten

(met een samenvatting in het Nederlands)

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"There's plenty of room at the bottom"
Richard Feynman, physicist, 1959

Preface

Preface

Glucocorticoids (GCs) are among the most frequently used anti-inflammatory and immunosuppressive agents in chronic inflammatory diseases. GCs have proven to be very powerful drugs in the treatment of rheumatic diseases. They quickly improve symptoms of rheumatoid arthritis (RA) such as pain and stiffness, and also decrease joint swelling and tenderness, and therefore GCs are useful both as temporary therapy, until the response to other slower-acting drugs (such as for instance methotrexate) is achieved, and as chronic therapy in severe RA that is not well controlled with use of standard treatment protocols. However, despite their beneficial effects, GCs can cause a whole range of adverse effects, including increased sensitivity for infections and gastrointestinal complications, hyperglycaemia, increased risk for cardiovascular events, and osteoporosis (1). The use of GCs is hampered by their highly unfavorable pharmacokinetic properties, i.e. rapid clearance and a large volume of distribution. This necessitates high and frequent dosing to maintain therapeutic levels at sites of inflammation, which increases the risk for serious adverse effects, especially upon long-term treatment (2). To improve their therapeutic index, GCs can be encapsulated in liposomes. This thesis focuses on the pharmaceutical optimization of liposomal GC formulations and their results in preclinical and clinical studies.

Liposomes have been extensively studied as targeted drug carrier systems in oncology and infectious diseases. Liposomes are small lipid bilayer vesicles enclosing an aqueous core in which water-soluble drugs can be enclosed. Liposomal physicochemical properties can be adapted to optimize penetration through biological barriers and retention at the site of administration, and to prevent premature degradation and toxicity to non-target tissues (3). Optimal liposomal properties depend on the administration route: large-sized liposomes show good retention upon local injection, small-sized liposomes are better suited to achieve passive targeting upon intravenous administration. PEGylation reduces the uptake of the liposomes by liver and spleen, and increases the circulation time (i.e long circulating liposomes, LCL), resulting in increased exposure at pathological target lesions (3). The phenomenon of passive targeting to pathological sites can be attributed to locally enhanced permeability of the vascular endothelium, allowing small-sized PEG-liposomes to extravasate and accumulate in the extravascular tissue, also referred to as the enhanced permeability and retention (EPR) effect (4). Additionally, targeting ligands can be attached to the liposomal surface to achieve selective delivery of the encapsulated drug to specific target cells in RA, referred to as active targeting. **Chapter 1** gives an overview of liposomal drug formulations studied in a preclinical setting as well as in clinical practice for the treatment of RA. It covers the use of liposomes as carriers for existing antirheumatic drugs as well as for new experimental agents in RA.

Prednisolone is often used to suppress joint inflammation in RA. Previously it has been shown preclinically that by encapsulating prednisolone in small-sized PEGylated liposomes,

the efficacy of the drug is increased dramatically, while side effects are minimized. The liposomes selectively accumulate in the inflamed areas, releasing prednisolone with such kinetics that the intensity and duration of the therapeutic effect is increased, while the occurrence of side effects is minimized (5). In **Chapter 2.1**, the influence of the type of GC (GCs differ in potency and clearance rate) is studied by comparing prednisolone-, dexamethasone- and budesonide- phosphate encapsulated in LCL regarding therapeutic activity and occurrence of adverse effects, in an attempt to further optimize the therapeutic index of liposomal GC in arthritis.

Despite their benefits, PEGylated liposomes are also known to cause hypersensitivity reactions in 5 -25% of the patients treated. It is thought that this is caused by activation of a part of the innate immune system, known as the complement system. Most of these complement-induced hypersensitivity reactions are transient and mild, but their intensity can be strong in hypersensitive patients (6). For further optimization of the liposomal GC formulation, it is investigated in **Chapter 2.2** whether the activation of the complement system by PEG-liposomes can be reduced by changing the 'PEGylation-profile' on the liposomal surface, such that the long circulating property is preserved while the risk of infusion reactions is minimized.

Liposomal formulations are usually aqueous dispersions. However, when dispersed in water, the phospholipids in the liposomal membrane can slowly become oxidized or hydrolyzed (7). This could result in fusion of liposomes, leakage of the enclosed drug compound, and structural transformations of the liposomes, which might influence their performance. Since dry products generally show a higher stability, we attempted to develop a dry liposomal formulation. However, the liposomal membrane is a delicate structure that can be easily disrupted and therefore needs to be protected during the drying process. In **Chapter 2.3** it is investigated whether hydroxypropyl-β-cyclodextrin (HPβCD), a cyclic oligosaccharide with some unique properties, is able to stabilize the liposomal membrane during spray-drying and freeze-drying of long circulating PEGylated liposomes.

Chapter 3 focuses on the preclinical and clinical results of liposomal GCs. In **Chapter 3.1** the efficacy and safety of liposomal administration of GC is tested in a mouse model for experimental arthritis. The suppression of the hormone regulation by the hypothalamic-pituitary-adrenal (HPA) axis is determined by measuring plasma corticosterone levels after administration of liposomal prednisolone or liposomal budesonide. As liposomal GCs have proven to be sufficiently safe in preclinical studies, **Chapter 3.2** describes the first clinical trial with a liposomal GC (i.e. prednisolone) in patients with active RA.

The performance of a liposomal formulation is critically dependent on the liposomal characteristics. Typically, small changes in one of these characteristics can have a huge impact on the in vivo behavior of the formulation. Therefore, proper characterization of the liposomal formulation is extremely important (8). However, regulatory requirements for this

specific class of medicinal products are still lacking. There is a need for a regulatory documentation structure for the registration and approval of liposomal drug products. The complexity of the regulation of this subgroup of nanomedicines, and recommendations for regulatory requirements are discussed in **Chapter 4**.

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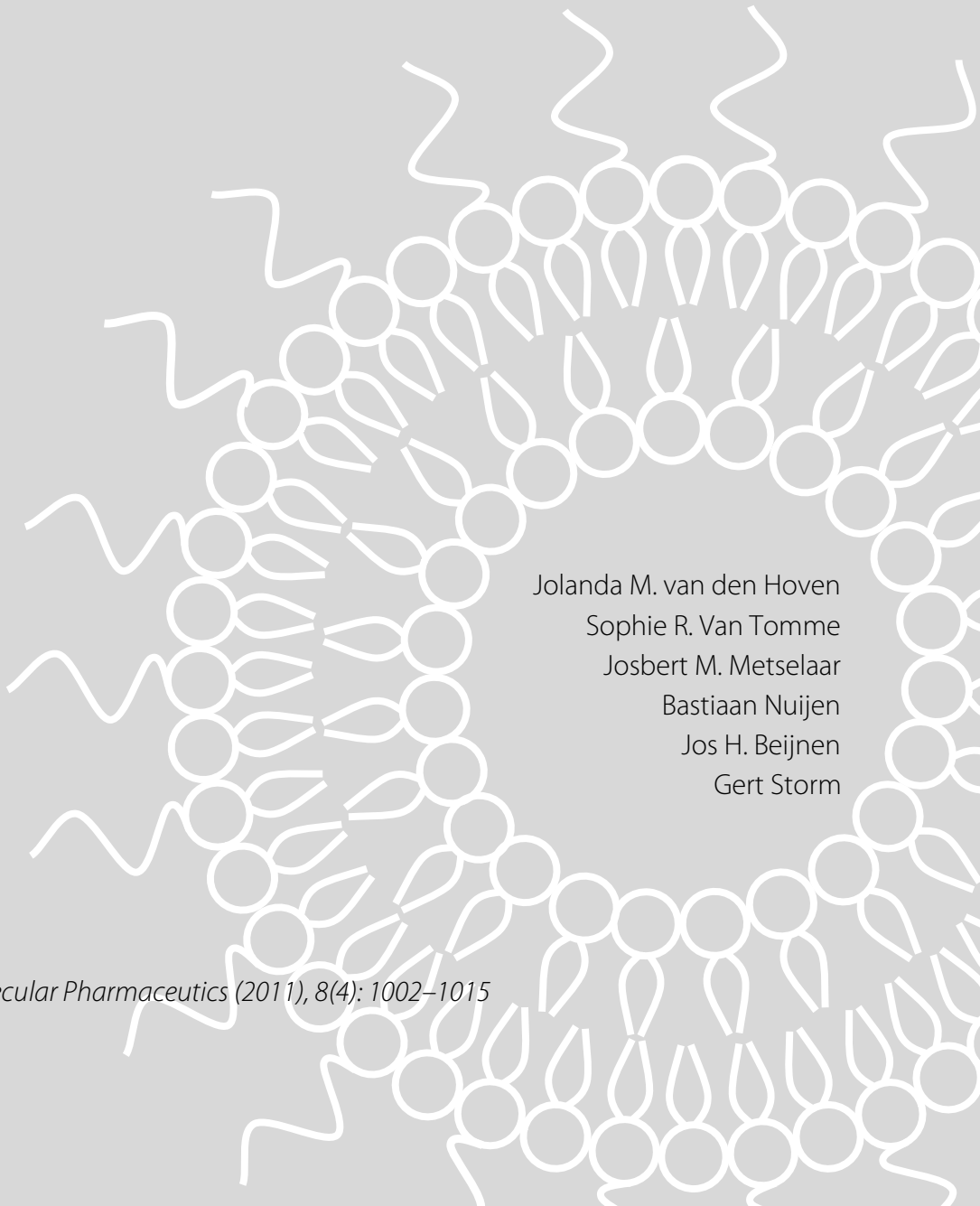
Table of contents

Chapter 1

Liposomal drug formulations in the treatment of rheumatoid arthritis



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Abstract

Liposomes have been extensively investigated as drug delivery systems in the treatment of rheumatoid arthritis (RA). Low bioavailability, high clearance rates and limited selectivity of several important drugs used for RA treatment require high and frequent dosing to achieve sufficient therapeutic efficacy. However, high doses also increase the risk for systemic side effects. The use of liposomes as drug carriers may increase the therapeutic index of these antirheumatic drugs. Liposomal physicochemical properties can be changed to optimize penetration through biological barriers and retention at the site of administration, and to prevent premature degradation and toxicity to non-target tissues. Optimal liposomal properties depend on the administration route: large-sized liposomes show good retention upon local injection, small-sized liposomes are better suited to achieve passive targeting. PEGylation reduces the uptake of the liposomes by liver and spleen, and increases the circulation time, resulting in increased localization at the inflamed site due to the enhanced permeability and retention (EPR) effect. Additionally liposomal surfaces can be modified to achieve selective delivery of the encapsulated drug to specific target cells in RA.

This review gives an overview of liposomal drug formulations studied in a preclinical setting as well as in clinical practice. It covers the use of liposomes for existing antirheumatic drugs as well as for new possible treatment strategies for RA. Both local administration of liposomal depot formulations as well as intravenous administration of passively and actively targeted liposomes are reviewed.

Introduction

RA is a systemic inflammatory disease characterized by chronic, progressive inflammation and gradual joint destruction. The primary target of the inflammatory process is the synovial tissue. Activated macrophages produce inflammatory cytokines that cause ongoing inflammation, joint swelling, bone erosion and cartilage damage. This results in pain, swelling, stiffness and functional impairment (1-4). Currently, there is no cure for RA (5). The goal of treatment is two-fold: to alleviate the burden of the patient and to minimize joint damage (3,4). Low bioavailability, high clearance rates and limited selectivity of several important drugs used for RA treatment make that high and frequent dosing is often required to reach satisfying therapeutic effects. However, such intensive treatment also increases the risk for the occurrence of severe side effects (2,6).

Liposomes have been investigated extensively as drug delivery vehicles to increase the therapeutic index of the encapsulated drug, and their versatility to accommodate a wide range of therapeutic agents has been demonstrated in preclinical and clinical settings (7). Liposomal physicochemical properties can be changed to optimize passage of biological barriers and retention at the target site, and to prevent premature degradation and toxicity to non-target tissues (8-14). Over the years liposomes have proven to be well tolerated carrier vehicles, as most liposomes consist of (semi)natural, biodegradable lipids (6). Despite these advantages, only few liposomal products have entered the market, with as leading examples: Doxil® (or Caelyx®, in Europe) and Myocet® (liposomal doxorubicin), and Ambisome® (liposomal amphotericin B).

Liposomal formulations can be applied locally as well as systemically. Local administration can be applied when the disorder is localized to only a single or a limited number of sites and when the site of pathology concerns a tissue that is readily accessible, as can be the case in RA. After systemic administration, the liposomal carrier system has to deliver the drug to the site of action. To achieve this, the so called 'passive targeting' phenomenon can be employed. Inflamed tissues are characterized by enhanced vascular permeability, which allows small, long-circulating drug carrier systems to extravasate at these sites. Subsequently they are retained in the extravascular space (often referred to as the EPR effect) (15,16), with a large portion being taken up by macrophages in the synovial layer (17), (18). Passive targeting and the EPR effect make the use of long-circulating liposomes attractive for improving the therapeutic index of anti-rheumatic drugs. Furthermore, by coupling of targeting structures to the liposomal membrane, specific cell populations can be targeted in the pathological site (also referred to as 'active targeting'). This strategy can potentially further improve the selectivity of the formulation.

This review provides an overview of liposomal drug formulations studied for use in the treatment of rheumatoid arthritis. Both local and systemic administration routes are addressed.

Rheumatoid arthritis

Clinical symptoms

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, often polyarticular, affecting multiple smaller and larger joints throughout the body. The prevalence is about 1%, and women are 3 times more prone to develop RA than men (1,2). Although considered an autoimmune disorder, the exact cause is unknown.

The primary target of the inflammatory process is the synovial tissue. Inflammation of the synovial tissue is characterized by formation of an oedematous and highly vascularized 'pannus-like' tissue that progressively invades and degrades underlying articular cartilage and bone (19). This pannus tissue originates from the synovial lining and consists of synovial macrophages, synovial fibroblasts and infiltrating inflammatory cells such as activated T and B lymphocytes. In addition to the invading pannus, the contents and volume of the synovial fluid are affected. Digestive enzymes (*e.g.* matrix metalloproteinases, MMPs) are secreted and attack surrounding tissue. Additionally, oxidative stress caused by reactive oxygen species (ROS) secreted by activated macrophages and other blood-derived cells is thought to contribute to tissue destruction (20). Joint and tissue destruction are the hallmarks of RA that ultimately culminates in immobility and deformity.

Current treatment strategies

Available treatment options for RA aim at symptomatic pain relief with non-steroidal anti-inflammatory drugs (NSAIDs) on the one hand, and slowing down disease activity and aiming for remission with disease modifying antirheumatic drugs (DMARDs) and corticosteroids on the other hand (2,3).

NSAIDs are drugs with analgesic, antipyretic and anti-inflammatory effects. Most NSAIDs act as non-selective inhibitors of the enzyme cyclooxygenase (COX-1 and COX-2), which catalyzes the formation of prostaglandins from arachidonic acid. Prostaglandins act as messenger molecules in the process of inflammation. Many NSAIDs display a short half-life after oral administration, demanding frequent and high dosing to achieve a full therapeutic effect in RA, thus increasing the risk for gastrointestinal side-effects (1,2).

DMARDs are effective in slowing down disease progression. The mechanism of action of most classic (synthetic) DMARDs in RA is still unclear. The antimetabolite methotrexate (MTX) is considered to be the most important and useful DMARD (21,22). It has an acceptable toxicity profile at low doses, and can be given orally. Other frequently used classic DMARDs are sulfasalazine, hydroxychloroquine, leflunomide, cyclosporin, intramuscular gold injections and azathioprine. More recently, biological DMARDs, such as tumor necrosis factor-alpha (TNFα) blockers and interleukin-1 (IL-1) blockers, have been

developed (5,23). These drugs appear to be highly effective as single agents as well as in combination with other DMARDs (24). Quite a few new biological DMARDs have been developed, and some are close to entering the market.

Glucocorticoids (GCs) are useful both as temporary therapy, until the response to DMARDs is achieved, and as chronic therapy in severe RA that is not well controlled with use of DMARDs. GCs are a class of steroid hormones with well-known immunosuppressive and anti-inflammatory effects, primarily as a result of their ability to modulate DNA transcription through binding to the cytosolic glucocorticoid receptor (25-28). At higher concentrations, GCs can also induce non-genomic anti-inflammatory and immunosuppressive effects (29). The use of GCs is hampered by their highly unfavorable pharmacokinetic properties, i.e. rapid clearance and a large volume of distribution, which necessitates high and frequent dosing to maintain therapeutic levels at sites of inflammation, which increases the risk for severe adverse effects, especially upon long-term treatment (26,30,31).

Currently, international recommendations for the treatment of RA are not available. Therefore, the European League Against Rheumatism (EULAR) aims to develop standards for this treatment. Based on five systematic literature reviews on available treatment options and related economic issues (22,24,28,32,33), three overarching principles and 15 recommendations were made, which are summarized in the treatment diagram in Figure 1 (34,35). Nowadays, treatment of RA often starts with the use of NSAIDs (2-4). However, since cartilage damage and bone erosions are known to occur already at early disease states, the EULAR recommends early start with DMARD treatment, skipping NSAID treatment (32). If possible a patient should start on MTX, otherwise leflunomide, sulfasalazine or injectable gold could be considered. (22,34,35). If a patient response to the therapy is insufficient, the treatment should be adapted as soon as possible (within 1-3 months). First, a change to another synthetic DMARD or, if prognostically poor factors are present, addition of a biological DMARD (especially a TNF-inhibitor) should be considered. If the first TNF-inhibitor fails, a second one can be tried. GCs can be used as initial, short term treatment, but their use should be tapered as soon as possible (22,24,28,32-35). It was shown that this treatment strategy was cost-effective (33). When the disease is stable for at least 12 months, slowly tapering of the biological and subsequently the synthetic DMARDs could be considered. However, discontinuation of DMARD therapy is associated with increased flare frequency, and moreover, remission is much harder to achieve after discontinuation of DMARD therapy. Therefore, tapering DMARDs should be performed cautiously and should be monitored strictly (34).

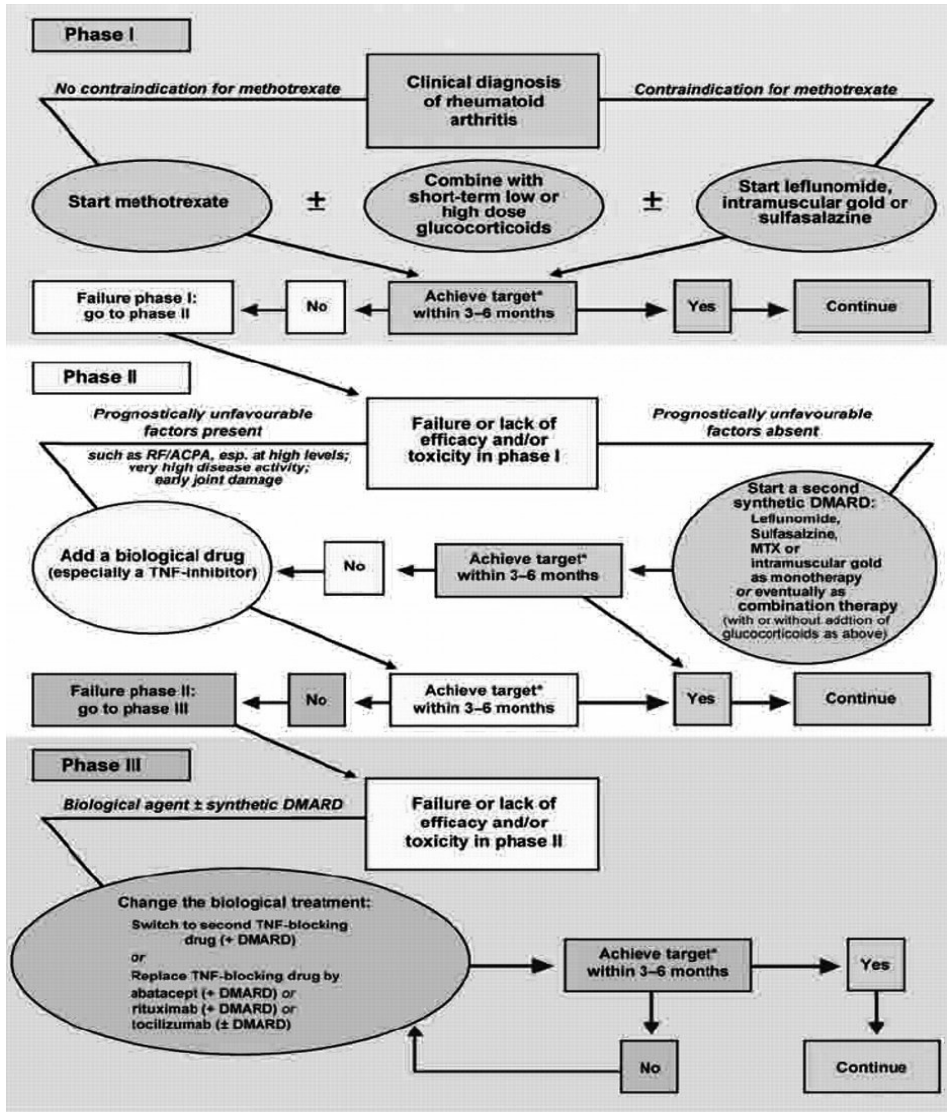

Figure 1: Treatment strategy based on the European League Against Rheumatism recommendations on rheumatoid arthritis management. DMARD, disease-modifying antirheumatic drug; MTX, methotrexate; RF/ACPA, rheumatoid factor/anti-citrullinated peptide antibodies; TNF, tumor necrosis factor. *The treatment target is clinical remission or, if remission is unlikely to be achievable, at least low disease activity. Adapted from Smolen, J. S. *et al.* EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. *Annals of the Rheumatic Diseases* 2010, *69*, 964-975.

Local administration

In some cases RA is restricted to only one or a few larger joints (commonly referred to as monoarthritis or oligoarthritis, respectively), which provides an opportunity for local treatment via the intra-articular (i.a.) route, resulting in a high local concentration with potentially minimal systemic exposure. The benefits of local treatment, however, can be limited by poor retention of the therapeutic agent in the joint (6,36,37). To improve and prolong drug exposure of the inflamed area after a single i.a. injection, liposomes have been studied as drug depot formulations after i.a. administration. To minimize systemic exposure, clearance of the drug from the joint after release from the liposome should be low.

Methotrexate

Already in 1988, Foong *et al.* tested an i.a. formulation of liposomal MTX (Table 1: A) in a rabbit model for arthritis. The clearance of free MTX from the joint is very fast after i.a. administration (38-40). To reduce this clearance, MTX was encapsulated in the aqueous interior of the liposomes. A 40-fold increase in drug retention in the joint was found, compared to injection of free MTX. However, only 4% of the liposomal MTX was associated with the synovium. Liposomal MTX was approximately 10-fold more effective in suppressing the development of arthritis compared to free MTX, when injected at the time of disease induction. Neither free, nor liposomal MTX was effective in the suppression of synovitis in established arthritis (40,41). Williams *et al.* state that this is because of rapid leakage of MTX from the liposomes, followed by rapid clearance from the joint. Therefore, they covalently coupled MTX to the phospholipid DMPE and incorporated this lipophilic derivative in the phospholipid bilayer of large multilamellar (MLV) and small unilamellar vesicles (SUV) (Table 1: B and C) (42). Fast leakage of the drug was successfully minimized by using the lipophilic derivative MTX-DMPE. A single i.a. injection of the MLV formulation in rats with experimental arthritis resulted in a rapid and sustained anti-inflammatory effect, superior to the effect of the SUV formulation. This was explained by the fact that the larger liposomes were more effectively retained in the inflamed joint compared to the smaller liposomes. Further experiments showed that a single i.a. injection of a comparable formulation of MLVs (Table 1: D) was able to reduce knee joint swelling to values comparable to non-arthritic knees in rats already after 7 days (43). It was not investigated if the biological activity of MTX was changed due to the chemical coupling to DMPE. Further research is needed to elucidate this possible change in biological activity.

NSAIDs

The NSAID diclofenac sodium (DFNa) has attracted increasing attention as a valuable agent in the treatment of RA, due to the quick onset of analgesic effects and anti-inflammatory properties. However, DFNa has a very short plasma half-life and can evoke adverse gastrointestinal side effects (44). Therefore, Türker *et al.* prepared various drug delivery systems for local administration of DFNa to the inflamed joints, to avoid systemic exposure and increase the local exposure time. Both liposomes and niosomes (*i.e.* vesicles prepared from non-ionic surfactants) of approximately 250 nm were compared to formulations that involve injectable hydrogels in which these vesicles were incorporated (lipogelosomes and niogelosomes, respectively) (45,46) (Table 1: E-H). 49-67% of the radio-labeled carrier was still present in the arthritic joint of rabbits 24h after i.a. injection of these formulations (45). The retention in the joint improved with increased viscosity of the formulation. Additionally, the release of DFNa in the most optimal formulation (Table 1: G using DPMC and C-940) is not only determined by release from the liposomes, but also by release of free drug from the surrounding gel network. Treatment with this DFNa-loaded lipogelosome formulation reduced joint swelling with 90% compared to the unaffected joint in arthritic rabbits. Cartilage damage and bone erosion were prevented. (46).

DFNa was also encapsulated in bioadhesive liposomes (BAL), carrying hyaluronan (HA) or collagen (COL) on their surface (Table 1: I and J). These liposomes have a high affinity for specific sites and molecules in the target area such as extracellular matrix, integrins, cartilage components and hyaluronan receptors, resulting in an increased retention of the liposomes in the joint. In rat osteoarthritis, a reduction of the inflammation of the knee joint over a time span of 17 days was seen after treatment with both types of liposomes. The most effective treatment was generated by combining DFNa and Dexamethasone (DEX) in HA-BAL, which yielded a reduction of the knee inflammation to 12.9% of its initial volume, as was calculated from the MRI data. No reduction in body weight was seen, pointing to acceptable tolerability of the formulation. (47)

Glucocorticoids

To increase the retention of GCs in the joint cavity, Lopez-Garcia *et al.* compared an i.a. injection of liposomal triamcinolone acetonide-21-palmitate (TAC-P) (Table 1: K) to i.a. injections of free triamcinolone acetonide (TAC) in rabbit arthritis. Due to the palmitate anchor, the drug is expected to be incorporated in the liposomal membrane. Whether the palmitate anchor has an effect on the biological activity of TAC has not been reported. The liposomal formulation induced increased retention in the articular cavity: 8 hours after treatment 38% of the liposomal TAC-P was still present in the joint cavity, while the free TAC was already completely cleared from the joint cavity within 1 hour. This retention correlates

with the increased reduction in paw diameter observed (13). Similar results were reported by Elron-Gross *et al*. for BAL containing DEX (47).

Bonanomi *et al.* entrapped the fatty acid-derivatized GC dexamethasone palmitate (DMP) in liposomes of different sizes (100 nm up to 30µm), lamellarity, charge and lipid composition (Table 1: L-Q), to improve the stability in the joint after i.a. administration (8,9). The retention of the various types of liposomes was compared to that of unencapsulated microcrystalline suspensions, containing both dexamethasone phosphate (DXP) and TAC, after a single i.a. injection in rabbits with arthritis. The retention in the synovium of healthy rabbits was optimal for liposomes with a mean diameter of more than 750 nm, with 6 times more intact large DMP-liposomes present in synovial fluid as compared to small DMP-liposomes 48 h after i.a. injection (8). The small DMP-liposomes showed a three times better anti-inflammatory response after 24 h compared to DXP/TAC-suspension in a three-times higher dose. The therapeutic effect of the large DMP-liposomes was not tested. None of the liposomal formulations suppressed the endogenous plasma cortisol.

Other therapeutic agents

Clodronate

Macrophages play a key role in RA, mainly by excreting a range of potent pro-inflammatory mediators and enzymes (48,49). Besides, they are responsible for clearance of liposomes by the mononuclear phagocyte system (MPS). Therefore, macrophages can be considered an interesting target cell population for liposomal drugs. Clodronate (dichloromethylene bisphosphonate) is a drug that induces apoptosis when delivered intracellularly into macrophages. Depletion of macrophages in the synovium by liposomal clodronate has been pursued experimentally to decrease the inflammation (49).

Van Lent *et al.* encapsulated clodronate in liposomes, for selective delivery of the drug to joint macrophages. This highly water-soluble drug cannot cross cell membranes in its free form and was therefore encapsulated in liposomes (Table 1: R), to achieve intracellular delivery in macrophages. After i.a. administration, clodronate was intracellularly released from the liposomes and induced apoptosis (50,51). Prophylactic depletion of local macrophages before induction of arthritis completely blocked immune cell infiltration and onset of arthritis in mice, showing the importance of macrophages in the initiation and maintenance of chronic arthritis (50). Barrera *et al.* studied this approach in RA patients and showed that macrophages were successfully depleted using liposomal clodronate (Table 1: S) and that this procedure was well-tolerated (52). Thus far, this is the only clinical study reported in literature that uses liposomal drug formulations.

Čeponis *et al.* demonstrated that weekly i.a. injections of low doses of liposomal clodronate (Table 1: T) had anti-inflammatory and joint-sparing effects in arthritic rabbits, without being

cytotoxic for cells. Significantly less TNFα was found in the synovium of liposomal clodronate-treated rabbits, as compared to untreated rabbits. However, the effect was only temporary and it did not prevent the occurrence of joint erosions over the long-term (53). Beside induction of apoptosis, low, non cytotoxic i.a. doses of liposomal clodronate appear to have chondroprotective and anti-inflammatory effects on damaged cartilage by the enhancement of levels of Cartilage Oligomeric Protein (COMP), an integral structure component of the cartilage matrix, as was shown in a rabbit model for arthritis by Gomez-Barrena *et al.* This means that liposomal clodronate can also have an important function in the repair potential of the cartilage, as it helps to strengthen the collagen network (54).

Lactoferrin

Liposomes have also been employed for the effective retention of macromolecular drugs in arthritic joints after i.a. administration. In RA, iron can potentially act as a catalyst in the production of damaging free radicals. Endogenous iron-binding proteins are often unable to bind all the iron that accumulates in synovial tissue and fluid. The enzyme Lactoferrin (Lf) is a glycoprotein that can bind free iron (55). Guillén *et al.* showed that periartricular injection (*i.e.* around the joint) of Lf significantly suppressed the inflammation. However, 75% of the injected Lf was cleared from the infected joint within 6 h and the anti-inflammatory effect lasted only for 3 days (56). Therefore, Trif *et al.* entrapped Lf in liposomes (Table 1: U-W), and compared the retention of ^{125}I-labeled liposomal Lf to the free protein after a single i.a. injection in arthritic mice (57,58). Free Lf was poorly retained in the joint, with 62% of the initial dose lost 2 h post-injection and only 2% remaining at 24 h (Figure 2). Entrapment in positively charged liposomes of 200 nm strongly increased the retention, with close to 50% of the initial dose still present at 6 h and 15% at 24 h post-injection (Figure 2). After a single i.a. injection of the positively charged liposome formulation, the arthritis severity decreased continuously over the full observation period of 12 days. Additionally, this liposomal Lf formulation reduced the pro-inflammatory cytokine production and increased the anti-inflammatory cytokine production compared, to free Lf (58). Entrapment in negatively charged liposomes did not improve the joint retention, and after 24h the Lf had already completely disappeared from the arthritic joint (57).

Biologicals

Recently a number of biological agents, the majority of which blocks TNFα, have been developed. One of these compounds is APO2L/TRAIL, that consists of 2 proteins related to the TNF-family, which both induce apotosis (59). I.a. injection of APO2L/TRAIL leads to apoptosis of synovial cells that contribute to joint destruction. However, the compound needs to associate with exosomes in the synovial fluid for its biological activity, and it was

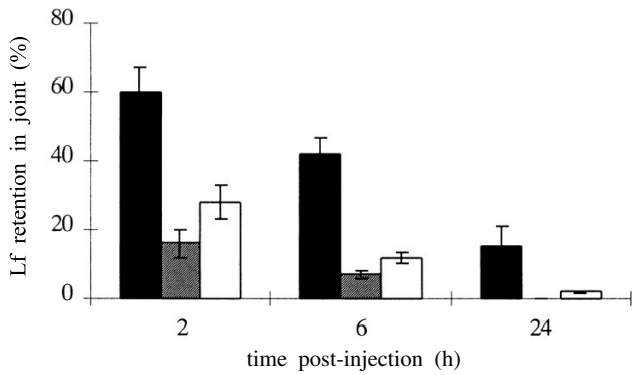

Figure 2: Joint retention (% of injected dose) of ^{125}I-labeled Lf after a single i.a. injection in arthritic mice. 1 mg ^{125}I-labeled Lf was administered as free protein □ (Lf (free)) or encapsulated in 200 nm charged liposomes (either in ■ Lf-L (positive) or in ■ Lf-L (negative)). Adapted from Trif, M. *et al.* Liposomes as possible carriers for lactoferrin in the local treatment of inflammatory diseases. *Experimental Biology and Medicine* 2001, *226*, 559-564 Copyright © (2011) Royal Society of Medicine Press, UK.

shown that exosome levels are extremely low in RA patients (60). Therefore Martinez-Lostao *et al.* conjugated APO2L/TRAIL to liposomes (Table 1: X). The liposomes can take over the function of the exosomes, mimicking the natural active form of APO2L/TRAIL. These APO2L/TRAIL liposomes were injected in the inflamed joint space in a mouse model, and this resulted in a reduction of synovial hyperplasia to almost normal values and 60% less joint inflammation, compared to only 30% for non-liposomal recombinant APO2L/TRAIL (61). Further studies regarding the effectiveness of this liposomal protein formulation are still ongoing.

It has been proposed that the anti-oxidant enzyme superoxide dismutase (SOD) protects cells from radical oxygen species (ROS), by catalyzing the dismutation of the toxic superoxide radical anion to oxygen and hydrogen peroxide (20). However, a major limitation of the therapeutic use of SOD is its short half-life of about 6 min after i.v. administration (62). Therefore, Simões *et al.* and Corvo *et al.* used liposomal formulations for local administration of SOD. Corvo *et al.* investigated the SOD delivery to the inflamed joint after subcutaneous injection (s.c.) of small-sized (110 nm, Table 1: Y) and larger-sized (450 nm, Table 1: Z) PEGylated liposomes in arthritic rats. The large-sized liposomes were retained at the site of injection to a twofold higher extend compared to the small-size liposomes. The uptake in the inflamed joint was 17 fold higher for the small-sized liposomes compared to large-sized liposomes. S.c. administration of small-sized liposomes appeared to be as effective as i.v administration, suggesting that the small-sized liposomes reach the circulation and are targeted to the inflamed area by the EPR effect (63).

Simões *et al.* focused on a novel route of administration: carrier-mediated transdermal transport with Transferosomes® (Tfs, Table 1: a). Tfs are ultradeformable mixed lipid liposomes, specifically developed for transdermal delivery of compounds (64). Tfs with a mean particle diameter of 150 nm were loaded with SOD and applied epicutaneously (e.c.)

on bare skin of arthritic rats (65). Daily e.c. application of SOD-Tfs (0.66 and 1.0 mg/kg body weight) appeared to have a larger anti-inflammatory effect compared to daily i.v. administration of long-circulating SOD-PEG-liposomes (0.066 mg/kg body weight). This paper provided for the first time evidence that transport of intact and therapeutically active enzymes from the healthy outer skin to the systemic circulation by entrapment in Tfs is possible.

Intravenous administration, passive targeting

When the target joint is not accessible for local administration, the drug may be targeted to the inflamed area after systemic administration. Inflamed tissues allow small, long-circulating drug carrier systems to extravasate by the EPR effect, referred to as passive targeting. (15,16). Traditional liposomes have a short blood circulation time after intravenous (i.v.) administration, due to rapid and efficient uptake by macrophages of the MPS, mainly those in the liver and spleen. Although targeting macrophages in liver and spleen might have a positive effect reducing the splenomegaly that is often seen in RA patients, rapid uptake of drug loaded liposomes by the MPS is not the prime consideration for using a liposomal drug formulation. For optimal use of the EPR effect to reach the target organ, i.e. the inflamed synovium, stable and long circulating liposomes are necessary. Long-circulating liposome formulations have been prepared by modifying the surface of liposomes with hydrophilic polymers such as poly(ethylene glycol) (PEG) (2,66) or, more recently, poly(vinyl alcohol) (PVA) (67) and poly(amino acids) (PAA) (68), all coatings which can effectively oppose uptake by macrophages of the MPS.

Methotrexate

Williams *et al.* encapsulated MTX-DMPE in the bilayers of 100 nm non-PEGylated liposomes (Table 1: C) and in 100 nm long-circulating PEG-liposomes (Table 1: b) and compared their therapeutic efficacy and toxicity in an arthritis model in rats. (43,69,70). It was anticipated that the long-circulating PEG-liposomes would accumulate in the inflamed joints, thereby delivering more MTX to the target tissues compared to the non-PEGylated ones. Surprisingly, the non-PEGylated liposomes showed considerable anti-inflammatory potency while the long-circulating PEG-liposomes did not reduce joint-swelling as compared to the saline-treated control group (69,70). Further testing showed that the non-PEGylated liposomes were more rapidly cleared from the circulation and taken up in the inflamed joint compared to the PEG-liposomes (1.5h vs. 24 h). This is reflected by the onset of the effect: joint swelling decreased already after 2 consecutive daily injections of non-PEGylated liposomes, while for the long-circulating PEG-liposomes this effect was started not earlier than day 6 after initiation of treatment (69). These findings were unexpected. The authors hypothesized that delivery to the macrophages is less effective for the PEG-coated liposomes, resulting in a later onset of the effect. (70). So in the short term, non-PEGylated

liposomes seem to reach the target more efficiently, but in the long term, the long-circulating ones are as efficient (69). The toxicity of liposomal MTX was reduced when compared to free MTX (70), indicating that the free MTX-levels in plasma after liposomal administration are quite low. No significant changes in red blood cell counts were observed after 4 days of treatment, but white blood cells and platelet counts were significantly lowered.

NSAIDs

Despite their proven therapeutic value, the high incidence of (gastrointestinal) side effects limits the use of NSAIDs in RA. Therefore, various groups have developed liposomes for local administration (8,9,13,47). For systemic administration, targeting the drug to the inflamed joints, however, only attempts for indomethacin have been made thus far.

Srinath *et al.* developed and optimized a liposomal formulation for indomethacin (Table 1: c). The lipophilic drug is incorporated in the lipid membrane, and its acid moiety has an electrostatic interaction with the amine moiety of the lipids in the membrane. As a result of this interaction, release from the liposome is quite slow. The liposomal formulation was significantly more effective in the inhibition of edema volume in rat models for arthritis, while the size and severity of occurring ulcers reduced compared to administration of free indomethacin (71). Further studies regarding the efficacy and safety of this formulation are ongoing.

Glucocorticoids

The potential of long-circulating liposomes to target GCs to sites of inflammation after i.v. administration, increasing their therapeutic index, was proven in several preclinical studies. Several GCs were tested, among which prednisolone disodium phosphate (PLP), dexamethasone disodium phosphate (DXP) (Table 1: d (72), Table 1: e (73-75) and budesonide disodium phosphate (BUP) (Table 1: d (72)). PLP was encapsulated in 100 nm long-circulating PEG-liposomes (Table 1: d) and tested in rat and mouse models of arthritis (17,76). A single i.v. dose of 10 mg/kg of free PLP did not result in a significant effect on paw inflammation, while the same single dose of PEGylated PLP liposomes resulted in the complete disappearance of the clinical signs of arthritis within 2-5 days (Figure 3). The anti-inflammatory effect lasted for one week, after which joint inflammation gradually reappeared. Daily i.v. injections of the same doses of free PLP for 5-7 consecutive days and single injections of the same dose of PLP encapsulated in small 100 nm non-PEG liposomes (Table 1: f) and large PEG-liposomes (450 nm diameter) were all much less effective (Figure 3). Both latter liposomal formulations showed enhanced uptake by macrophages in the liver and spleen and diminished accumulation in inflamed paws.

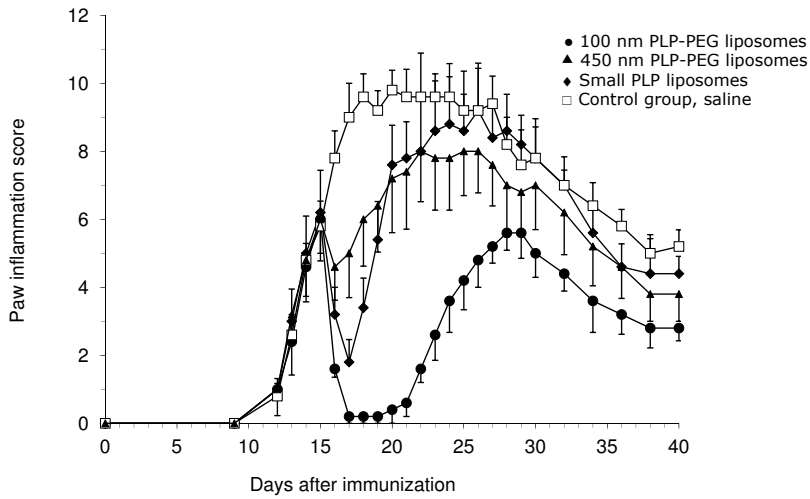

Figure 3: Therapeutic effect of a single i.v. injection of 10 mg/kg PLP encapsulated in long-circulating PEG-liposomes (●100 nm PLP-PEG-L), large PEG-liposomes (▲450 nm PLP-PEG-L) and 100 nm non-PEGylated liposomes (♦PLP-L) as compared to treatment with saline as a control (□) in arthritic rats. Arrow indicates treatment day. Adapted from Metselaar, J. M. *et al.* Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating lipsomes. *Arthritis & Rheumatism* 2003, *48*, 2059-2066, used with permission.

Given the fact that PEG is not easily degraded, it cannot be excluded that PEG accumulates intracellularly where it could interfere with cellular processes (77). Therefore biodegradable alternatives to PEG have been developed, like poly(hydroxyethyl L-asparagine) (PHEA) (78), PVA (67) and PAA (68). The therapeutic activity of PLP encapsulated in biodegradable PHEA-liposomes (Table 1: g) in arthritic rats was equal to that of PLP encapsulated in PEG-liposomes at the same dose (78).

Compared to liposomal PLP, liposomal DXP had a comparable anti-inflammatory effect at a five times lower dose (2 mg/kg DXP vs. 10 mg/kg PLP) in arthritic rats (72). Liposomal treatment with DXP could reduce the dose of DXP by a factor 3-10 compared to free DXP (74,75).

Treatment with BUP in PEG-liposomes at a dose of 1 mg/kg was as effective as DXP in PEG-liposomes at a dose of 2 mg/kg in arthritic rats, while showing hardly any systemic adverse events. Therefore BUP might be a promising candidate for liposomal encapsulation (72).

Avnir *et al.* hypothesized that a higher encapsulation efficiency and a higher molar drug to lipid ratio might provide a formulation with superior overall characteristics (79). Amphipathic weak acid pro-drugs methylprednisolone hemisuccinate (MPHS) or betamethasone hemisuccinate (BMHS) were loaded into 85 nm PEG-liposomes using a remote loading technique described by Clerc *et al.* (80). The pharmacokinetics and the anti-inflammatory

effect of these remote loaded liposomes (Table 1: h) were compared to free MPHS and BMHS in rats with arthritis and in a beagle dog (79). The pharmacokinetic results obtained in both animal species were very similar. Treatment with both liposomal formulations resulted in complete remission of inflammation three days after the first injection, after which joint swelling increased within two weeks to values comparable to control animals (79).

Other therapeutic strategies using passive targeting of liposomes

Clodronate

As discussed above, bisphosphonates can be used for depletion of local macrophages to reduce the inflammation in RA. Initial experiments with i.v. administration of liposomal formulations focused on the influence of liposome size and composition. After i.v. injection, small liposomes (in the 100 nm range) were shown to accumulate to a significantly greater extent than large liposomes (in the µm range) in inflamed joints (81). Larger-sized clodronate liposomes reduced joint swelling, but did not prevent joint destruction (82,83). This result was confirmed by an experiment by Kinne *et al*., who showed that multilamellar clodronate-containing vesicles (Table 1: i) induced depletion of macrophages in the liver and spleen, but had no effect on the macrophages in the synovial layer, while a significant reduction in joint swelling was seen. (83). Highton *et al*. tested large-sized clodronate liposomes (Table 1: j) in a sheep model for arthritis. In this model no reduction of joint swelling was seen, although they did show that the liposomes reached the inflamed synovium (84).

To more specifically target the macrophages in the inflamed joint, Love *et al*. developed small-sized clodronate liposomes. Depletion of macrophages in the joint would reduce the inflammation (85). Richards *et al*. tested these clodronate-containing small-sized liposomes (Table 1: k) in arthritic rats for their ability to deplete synovial macrophages, and compared them to larger multilamellar liposomes containing clodronate (Table 1: m) after i.v. injection (86). A single i.v. dose of small-sized liposomes was more effective than larger-sized ones, sustaining a significant reduction in knee swelling for up to 7 days. The limited efficacy of the larger-sized liposomes was attributed to its strong localization in the MPS, where it effectively depleted the hepatosplenic macrophages. Large liposomes thus failed to accumulate in inflamed joints after systemic administration (86). Richards *et al.* later assessed the prophylactic effect of liposomal clodronate on the onset of arthritis when treated 10 days after disease induction (87). The local macrophage elimination that resulted from administration of small-sized clodronate liposomes significantly suppressed the development of arthritis and induced a significant reduction in synovial levels of pro-inflammatory interleukins, TNF-α and MMPs (87).

Superoxide dismutase

As already addressed, SOD can be used to protect cells against ROS, but it has a plasma half-life of about 6 min. To avoid rapid clearance via the kidneys, Corvo *et al.* encapsulated SOD in 3 types of radiolabeled liposomes (Table 1: n and o) (88). Additionally, they strongly improved the encapsulation efficiency of SOD into PEG-liposomes (Table 1: o) by transiently lowering the pH to 3.3 (below the isoelectric point of the protein), yielding a positively charged protein that showed enhanced interaction with the negatively charged lipids during the formation of the liposomes. Readjusting the pH to 5.6 fully restored the enzymatic activity of SOD (89). These PEGylated liposomes were subsequently compared for their therapeutic activity to non-PEGylated liposomes, after i.v. administration to rats with arthritis, in a dose range of 33 to 363 μg/rat (89). The PEGylated liposomes resulted in 3-fold (200 nm) and 8-fold (100 nm) higher blood levels than the positively charged stearylamine-containing liposomes, 24h after administration (89). The prolonged circulation time and small size resulted in a strong accumulation in the inflamed areas. However, even repeated daily administration of the optimal formulation during the course of disease development was not able to completely eliminate joint swelling (63).

Gaspar *et al.* state that this limited therapeutic efficacy of liposomal SOD could be caused by a limitation in the extent and/or rate of release in the inflamed area. Therefore, they developed a new formulation of SOD, in which they covalently conjugated multiple fatty acid chains to SOD, rendering a more lipophilic acylated SOD, with only a 10% reduction of the enzymatic activity (90). When incorporated in PEG-liposomes, it retained its enzymatic activity, while being partly present at the liposomal surface. This can be considered an advantage, because release is not longer needed for therapeutic activity (90). The therapeutic potential of these so-called enzymosomes (liposomes containing surface-presented SOD, Table 1: n and o) were compared with PEGylated and non-PEGylated (SA containing) liposomes containing conventional SOD within their interior at doses of 33, 165 and 363 μg per animal in arthritic rats (Table 1: n and o) (91). As expected, the circulation time of the PEGylated liposomes was longer than for the non-PEGylated ones, reflected also by a lower hepatosplenic uptake. The therapeutic benefit of enzymosomes presenting modified SOD on their surface was a faster onset of anti-inflammatory activity after i.v. injection, indeed suggesting that surface-exposed SOD exerted its enzymatic effect without the need to release the encapsulated SOD (91).

Therapeutic strategies using activation after injection

In photodynamic therapy a photosensitizing drug is delivered to its site of action where it is activated using a selective wavelength of laser light. Upon activation, the photosensitizing agent forms short lived oxygen derived species that induce damage, anoxia and promote apoptosis of the affected cells. Chowdhary *et al.* treated arthritic rabbits i.v. with the

photosensitizing drug BPD-Verteporfin® (liposomal BPD-MA) to determine the time and dose dependency for reduction of joint inflammation. BPD-MA was delivered by the liposomes to the inflamed synovium as well as the surrounding tissues with a high degree of vascularization, but was also rapidly cleared from the synovium. It was concluded that to treat the synovium, early light exposure is needed. Apoptosis was seen in 27% of the cells in the synovium, making targeted photodynamic therapy a possible treatment strategy in RA (92).

Another strategy tested in RA is Boron Neutron Capture Therapy (BNCT). BNCT is a form of radiotherapy that depends on the interaction of slow neutrons, applied by a neutron beam, with ^{10}B that was injected to the patient. Upon absorption of a neutron, the heavier ^{11}B disintegrates into a lithium nucleus (^{7}Li) and an alpha particle, without producing other types of ionizing radiation. These particles cause ionizations over a length of only one cell diameter, thereby sparing the surrounding tissues. Watson-Clark *et al*. targeted the boron to the inflamed synovium using liposomes. For this type of therapy, at least 15 µg of boron per gram target tissue is needed. Liposomal formulations containing different amounts of boron species embedded in the vesicle bilayer (Table 1: p and q) or encapsulated in the aqueous core (Table 1: r and s) were compared. The liposomes were administered i.v. in a rat model for arthritis. Boron was delivered to the synovium and retained there, resulting in a final boron concentration of 26 µg per g tissue, and a synovium/blood ratio of 2.0 after 48 hours, after which the boron level slowly decreases to 14 µg per g tissue after 96 h (Table 1: s). Further research is needed to test the efficacy and safety of this approach (93). However, during irradiation, a high number of neutrons have to be directed to the target. Currently, only nuclear reactors are able to provide such a neutron beam and the energy spectra of the neutron beams available can differ considerably, which limits the clinical application of BNCT. For a multicenter clinical trial to be conducted the beam energy has to be standardized, which currently seems very difficult to achieve. (94,95)

Small interfering RNA (siRNA)

In living cells RNA interference is a natural occurring mechanism to control the expression of genes. This mechanism is exploited by delivery of siRNA into target cells, to stop the production of a certain protein. In RA, TNFα is one of the most prominent cytokines, and by silencing TNFα transcription in macrophages, the disease activity can be reduced. To specifically deliver siRNA designed to silence TNFα, to the macrophages in the inflamed joints, a liposomal formulation was developed by Khoury *et al.* (Table 1: t). Complete cure was seen in arthritic mice after i.v. treatment with 10 µg siRNA encapsulated in cationic liposomes. Due to their large size (2-3 µm) a large part of the liposomes was targeted to liver and spleen, but despite this, the inflamed joints were also targeted. The TNFα secretion was decreased by 50-70%, resulting in a reduction of the incidence and severity of the inflammation (96). Beside TNFα, Khoury *et al.* also encapsulated siRNA designed to silence IL-

1, IL-6 and IL-18 (Table 1: t). I.V. administration of the liposomes resulted in a delay of the onset of the disease, a reduction of the incidence and severity of the inflammation and an inhibition of proinflammatory gene expression (both local and systemic). The best results were obtained when the three different siRNA sequences were combined in the same liposome, attacking different pathways of disease development. This combination was as effective in reducing paw swelling and arthritis severity in mice as the liposomal formulation containing siRNA against TNFα (97).

Intravenous administration, active targeting

Targeting ligands can be coupled to the liposomal surface to enable binding to receptors (over)expressed at the target site, referred to as active targeting. In RA, macrophages are an obvious target, but as described above, these cells can be targeted using passive targeting mechanisms. Besides passive targeting, several actively targeted liposomal formulations have been designed to target other cell types that play important roles in RA: endothelial cells and T-cells.

Active targeting to vascular endothelial cells (VECs)

VECs at the inflamed site play a crucial role in inflammatory processes. At the same time, they provide easy access to i.v. administered drug carrier formulations. Therefore, VECs are an interesting target for the treatment of RA.

The cell adhesion molecule E-selectin is a suitable target molecule because it is selectively expressed on VECs activated by cytokines at sites of inflammation (98,99). To target E-selectin, the tetrasaccharide Sialyl-Lewis X (SLX), the natural ligand for E-selectin, was conjugated to the surface of 100 nm liposomes, containing a fluorescent substance (Table 1: u). The accumulation of SLX-liposomes in sites of inflammation in arthritic mice was compared to liposomes lacking the ligand or bearing an irrelevant ligand. SLX-liposomes accumulated in sites of inflammation to a greater extent compared to control liposomes, as was visualized using scanning fluorescent microscopy. It is thought that this selective accumulation occurs via the same pathway as the accumulation of leucocytes in inflamed areas, since leucocytes also express SLX on their surface. The anti-inflammatory effect of drugs loaded into these liposomes has not yet been investigated (99).

Another way to target VECs is by exploiting the strong up-regulation of the integrin αvβ3 on angiogenic VECs at sites of inflammation (100). Cyclic Arg-Gly-Asp sequence-containing peptides (cRGD) have been developed as specific high-affinity ligands for these αvβ3 integrins (101). Koning *et al.* encapsulated DXP in 100 nm PEG-liposomes, with cyclic RGD peptides covalently attached to the distal ends of the PEG chains (Table 1: v) (100). After i.v. administration to rats at the onset of arthritis, the RGD-PEG- liposomes were cleared more rapidly from the circulation compared to the control PEG-liposomes. However, 3-fold higher

accumulation at sites of inflammation compared to the control PEG-liposomes was achieved. This suggests that the specific targeting mechanism is more effective than the EPR effect used in passive targeting in reaching the target site in an early state of the arthritis (100). A single i.v. injection of DXP loaded into these RGD-PEG-liposomes had a strong and prolonged anti-inflammatory effect in rats with experimental arthritis, which was by far more efficacious than DXP loaded into passively targeting PEG-liposomes. This indicates that active targeting to VECs at the inflamed site might be a favorable way to treat RA (100).

Active targeting of auto-aggressive T-cells

It is hypothesized that, besides macrophages, autoreactive T-cells play a major role in the etiology of RA. Autoreactive T-cells secrete cytokines that activate synovial macrophages and fibroblasts, and thereby contribute to the inflammatory process. By blocking these T-cells selectively, the production of cytokines can be reduced (102). Upon activation, $CD4^+$T-cells in RA express the activation marker CD134. These auto-aggressive $CD4^+$T cells are mainly present in the synovial fluid in RA patients. To selectively block these T-cells, Boot *et al.* actively targeted activated auto-aggressive $CD4^+$ T-cells that show upregulation of the expression of surface marker CD134 (103). PEG-liposomes were coated with monoclonal antibodies against CD134 (Table 1: w) and were tested in a rat model for arthritis. Although the anti-CD134-liposomes were shown to specifically bind to the activated T-cells, they were not internalized. This unexpected finding led to the use of a fatty-acid-derivatized drug to enable lipid-coupled drug transfer between the liposomal membrane and the cell membrane of the target T-cell to achieve intracellular drug delivery. In this study dipalmitate-5'-fluorodeoxyuridine (FudR-dP) was used. Indeed, the severity of the developing arthritis was reduced, albeit only to a moderate extent (103).

Concluding remarks

Extensive attention has been given to the concept of liposomes as drug carriers to improve the therapeutic index of drugs. However, with respect to the application of liposomal drug delivery in the treatment of RA, the literature is still limited and fragmentary, and lacks systematic and comparative studies. Nevertheless, as clearly evidenced by the literature reviewed in this contribution, liposomal carriers can be very functional to improve the therapeutic performance of anti-inflammatory agents in RA, either by introducing a depot (local administration) or by attaining site specific drug targeting (intravenous administration). It is obvious that liposomes compete with other delivery systems in this field, but they are particularly attractive by virtue of their great flexibility in terms of composition, physicochemical characteristics and ability to accommodate a wide spectrum of drug molecules. Large-sized liposomes are particularly attractive to achieve slow release effects upon local administration. When administered locally, liposomal drugs have

demonstrated to be more effectively retained compared to the free drug. Local treatment with a liposomal formulation could be indicated when the disease is limited to only a few, readily accessible joints. Small-sized liposomes are better suited to achieve targeting after intravenous administration. Surface modifications can be introduced to further improve target localization by prolonging the circulation time (passive targeting) and/or by interacting with specific target cell receptors (active targeting).

Overviewing the preclinical literature, particularly liposomal formulations of methotrexate and glucocorticoids appear to be promising candidates for further translational studies into their role as therapeutic intervention when exacerbations occur. In case of liposomal methotrexate, the advantage would be that the patient does not have to switch to a therapy with another drug molecule. In the current recommendations, the use of GC in the treatment of RA is to be kept as low as possible (28,34). However, the publications on liposomal GCs reviewed here suggests that liposomal formulations could change this point of view.

Traditional therapies in RA have consisted of anti-inflammatory and immunomodulatory agents, and both therapeutic classes may exert undesirable side effects. NSAIDs, systemic glucocorticoids and methotrexate or other DMARDs, are known to cause renal, gastrointestinal, neurologic, hematologic or immunologic toxicities. Also with the newer biological therapeutics, there is a need to improve their side effect profiles. The use of liposomes represents an attractive strategy to overcome toxicity problems associated with these traditional and newer therapeutic agents.

Currently, many promising new therapeutic agents are entering the market or are in late phase of clinical studies. Most of these are biologicals, specifically binding to pro-inflammatory cytokines and other proteins, like infliximab, etanercept, cetolizumab pegol, golimumab and adalimumab (all anti-TNFα), tocilizumab (anti-IL-6), anakinra (anti-IL-1), abatacept (anti-CD28) and rituximab (anti-CD20). However, despite their success, the most important issue with the biologicals is the price, though the costs will likely decrease over time (2,33,34). Also a range of new small-molecular agents are in development, such as the kinase inhibitors INCB-28050, tasocitinib and fostamatinib disodium, which are currently in Phase II and III trials (104).

With all these successful new therapies lining up, an important question to address here is whether or not there will still be a market for novel liposomal formulations in RA. While this review shows that liposomes can be highly functional in the target therapy of RA, no liposomal formulations for the treatment of RA have been marketed yet, nor are there any in clinical development at this moment. To date, only one clinical study has been published (52). Apparently, the translation of the reported preclinical successes into clinical application is not straightforward. This may partly be explained by the fact that industry prefers novel therapeutic products and new chemical entities over improved liposomal reformulations of

existing (often generic) therapeutic agents. Also, in relation to the generic compound in its free form, the market price of its improved liposomal equivalent is likely going to be several times higher, and it is questionable whether or not the potential improvement of the therapeutic index by the liposomal formulation is going to be sufficiently valuable from a clinical perspective to allow for such a price premium. Most of the current literature demonstrates improved efficacy when a drug is administered in a liposomal formulation as compared to the free drug. However, potential toxicity issues have not been explored in detail. And yet, this may become the decisive factor for the application of liposomal formulations in the clinical setting. And lastly, most liposomal formulations need to be given i.v. or i.a., and therefore need a hospital setting. Hospitalization entails expenses on the one hand, and may be a burden for the patient on the other hand, which can be considered a significant hurdle by drug marketers.

Clearly, if there were a place for novel i.v. liposomal products in the treatment of RA, it should be in Phase III of the treatment strategy as outlined by the EULAR (Figure 1) besides the biologicals, but only if a clear advantage can be shown at the level of therapeutic index and/or at the level of treatment costs. There might also be a more restricted place for novel i.v. liposomal products in Phase II of the EULAR RA treatment strategy if the liposomal product can be used in an intervention setting to induce a remission during a phase of active RA in a patient who is otherwise stable on relatively cheap generic DMARDs. In this situation an effective liposomal product may help keeping a patient in this phase, preventing the switch to the more expensive third phase.

Liposomal formulations for local, intra-articular RA therapy fall a bit outside this EULAR treatment strategy discussion. Here the question is purely whether or not liposomal encapsulation of the agent results in an increased and prolonged effect of the incorporated drug without causing any safety issues. The market for such a product is likely limited to only those cases in which only one or a few joints are severely affected.

Concluding, while the advances in the field of liposomal drug delivery in RA as reviewed in this contribution are encouraging, one should be careful to claim great expectations on the basis of these achievements for the future of the management of RA. Clearly, additional (pre)clinical research is mandatory to demonstrate clinical and commercial application in RA therapy.

Table 1: Liposomal drug formulations in the treatment of RA

Label	Type	Drug	ø (nm)	Composition (molar ratio)	Route of administration	Animal model	Reference
A	Not defined	MTX	1070	EPC/CHOL/DCP (5:5:1)	i.a.	Rabbit AIA	(40,41)
B	MLV	MTX-DMPE	1200	EL/CHOL/PA (7:2:1)	i.a	Rat AIA	(42)
C	SUV	MTX-DMPE	100	EL/CHOL/PA (7:2:1)	i.a, i.v	Rat AIA	(42,105)
D	MLV	MTX-DMPE	1,200	POPC/CHOL/DMGP (7:2:1)	i.a.	Rat AIA	(43)
E	Not defined	DFNa	235 or 242	DMPC or DSPC/CHOL/DCP (7:1:2)	i.a.	Rabbit AIA	(45,46)
F	Niosomes	DFNa	270	SUR I/CHOL/DCP (7:1:2)	i.a.	Rabbit AIA	
G	Lipogelosomes	DFNa	235 or 242	DMPC or DSPC/CHOL/DCP (7:1:2) mixed 1:1 (w:w) with 1% C-940 or CMC-Na	i.a.	Rabbit AIA	
H	Niogelosomes	DFNa	270	SUR I/CHOL/DCP (7:1:2) mixed 1:1 (w:w) with 1% C-940 or CMC-Na	i.a.	Rabbit AIA	
I	HA-BAL (MLV)	DEX, DFNa	Unknown	SPC/DPPE (95:5)+HA	i.a.	Monosodium iodoacetate model of OA in rats	(47)
J	COL-BAL (MLV)	DEX, DFNa	Unknown	SPC/DPPE (95:5)+COL	i.a.	Monosodium iodoacetate model of OA in rats	
K	Not defined	TAC	Unknown	DPPC/CHOL/PA (8:3:1)	i.a.	Carrageenan induced paw edema model in rats	(13)
L	Not defined	DMP	160	EPC/octyl glucoside mixtures (10:51.2)	i.a.	Rabbit AIA	(8)
M	OLV	DMP	4500	EPC/octyl glucoside mixtures (10:51.2)	i.a.	Rabbit AIA	(9)
N	OLV	DMP	750	EPC/PA /C8E4 (10:1:56.2)	i.a.	Rabbit AIA	(8,9)
O	MLV	DMP	950	EPC/SA /C8E4 (10:1:56.2)	i.a.	Rabbit AIA	(9)
P	MLV	DMP	2000	EPC/PA (10:1)	i.a.	Rabbit AIA	
Q	MLV	DMP	9000	DPPC/PA (10:1)	i.a.	Rabbit AIA	
R	Not defined	Clodronate	1000	PC/CHOL (~10:1 w/w)	i.a.	Healthy mice, mouse CIA	(50,51)
S	Not defined	Clodronate	120-160	PC/CHOL (unknown ratio)	i.a.	RA patients (human)	(52)
T	Not defined	Clodronate	Unknown	DSPG/DSPC/CHOL (unknown ratio)	i.a.	Rabbit AIA	(53)
U	Liposomes(+)	Lactoferrin	200	PC/CHOL/PS (5:5:1)	i.a.	Mouse CIA	(57,58)
V	Liposomes(+)	Lactoferrin	200	DOPE/CHEMS (6:4)	i.a.	Mouse CIA	
W	Liposomes(-)	Lactoferrin	200	DPPE/CHOL/SA (5:5:1)	i.a.	Mouse CIA	
X	LUV	APO2L/TRAIL	Unknown	EPC/SM/CHOL/DOGS-NTA (unknown ratio)	i.a.	Rabbit AIA	(61)
Y	Liposomes (-)	SOD	110 nm	EPC/DSPE-PEG/CHOL/SA (unknown ratio)	s.c.	Rat AA	(63)
Z	Liposomes (-)	SOD	450 nm	EPC/DSPE-PEG/CHOL/SA (unknown ratio)	s.c.	Rat AA	
a	Transferrosomes	SOD	150	SPC/sodium cholate (3.75:1)	e.c.	Rat AA	(64,65)
b	PEG-liposomes	MTX-DMPE	100	DSPC/CHOL/DSPE-PEG (10:5:1)	i.v.	Healthy rats, Rat CIA	(69,70)
c	LUV	Indomethacin	100	EPC/CHOL/SA (1:0.5:0.1)	i.p.	Carrageenan induced paw edema model in rats	(71)

d	PEG-liposomes	PLP, DXP, BUP	100/450	DPPC/CHOL/DSPE-PEG2000 (1.85:1.0:0.15)	i.v.	Rat AA, rat CIA	(17,72,76)
e	Non-PEG-Liposomes	DXP	285-310 nm	DPPC/DPPG/CHOL (50:10:40 mol%)	i.v.	Rat AIA, rat CIA	(73-75)
f	Liposomes	PLP	100	DPPC/CHOL (2.0:1.0)	i.v.	Rat AA	(76)
g	PHEA-liposomes	PLP	150	DPPC/CHOL/PHEA-lipid-conjugate (1.85:1.0:0.15)	i.v.	Rat AA	(78)
h	PEG-liposomes	MPHS, BMHS	85	HSPC/CHOL/DSPE-PEG2000 (55:40:5)	i.v.	Rat AIA, healthy beagle dog	(79)
i	MLV	Clodronate	unknown, large-sized	PEG-S/ SDS/CHOL (4:1:4)	i.v.	Rat AA	(83)
j	MLV	Clodronate	Unknown, large-sized	CHOL/PC/PS (40:80:8 w/w/w)	i.v.	Sheep AIA	(84)
k	Not defined	Clodronate	100	EPC/CHOL/DPPA (7:7:1)	i.v.	Rat AIA, SCW induced arthritis model in rats	(86,87)
m	MLV	Clodronate	Unknown, larger-sized	EPC/CHOL (2:1)	i.v.	Rat AIA	(86)
n	Liposomes(+)	SOD	200 / 100 nm	EPC/CHOL/SA (7:2:1)	i.v.	Rat AA	(88,89,91)
o	PEG-liposomes	SOD	110 / 200 nm	EPC/CHOL/DSPE-PEG2000 (1.85:1.0:0.15)	i.v.	Rat AA	
p	Not defined	-	65 nm	DSPC/CHOL/Lipophillic boron species (3:3:1)	i.v.	Rat CIA	(93)
q	Not defined	-	88 nm	DSPC/CHOL/Lipophillic boron species (3:3:2)	i.v.	Rat CIA	
r	Not defined	-	65 nm	DSPC/CHOL/Hydrophillic boron species (3:3:1)	i.v.	Rat CIA	
s	Not defined	-	88 nm	DSPC/CHOL/Hydrophillic boron species (3:3:2)	i.v.	Rat CIA	
t	Liposomes (+)	siRNA targeting TNFα, IL-1, IL-6 and/or IL-18	2000-3000 nm	DOPE/Cationic lipid RPR209120/DNA-carier (unknown ratio)	i.v.	Mouse CIA	(96,97)
u	Not defined	-	100	DPPC/CHOL/DCP/ganglioside/ DPPE/sodium cholate (16.8:10.1:1.8:14.6:2.3:46.9 w/w/w/w/w/w) mixed 1:1 with DTSSP, +SLX (up to various densities)	i.v.	mAb induced arthritis in mice	(99)
v	PEG-liposomes	DXP	100	DPPC/CHOL/DSPE-PEG2000/DSPE-PEG2000-maleimide (1.85:1.0:0.075:0.075)	i.v.	Rat AIA	(100)
w	PEG-liposomes	FUdR-dP	100-200	EPC/CHOL/DSPE-PEG2000/ DSPE-PEG2000-maleimide (2:1:0.075:0.075)	s.c.	Rat AA	(103)

List of abbreviations

AA adjuvant arthritis, **Ab** antibody, **AIA** antigen-induced arthritis, **BAL** bioadhesive liposomes, **BMHS** betamethasone hemisuccinate, **BNCT** boron neutron capture therapy, **BPD-MA** BPD-Verteporfin®, **BSP** betamethasone sodium phosphate, **BUP** budesonide disodium phosphate, **C8E4** N-octyltetraoxyethylene, **C-940** Carbopol 940 (crosslinked PAA), **Cationic lipid RPR209120** (2-(3-[Bis-(3-amino-propyl)-amino]-propylamino)-N-ditetradecylcarbamoylme-thyl-acetamide, **CHEMS** cholesterol-hemisuccinate, **CHOL** cholesterol, **CIA** collagen-(type II)-induced arthritis, **CMC-Na** carboxymethyl cellulose, **COL** collagen, **COMP** cartilage oligomeric protein, **COX** cyclooxygenase, **cRGD** cyclic Arg-Gly-Asp motif containing peptide, **DCP** diacethyl phosphate, **DEX** dexamethasone, **DFNa** diclofenac sodium, **DMARD** disease modifying antirheumatic drug, **DMGP** 1,2-dimyristoyl-sn-glycero-3-phosphate, **DMP** dexamethasone palmitate, **DMPC** dimyristoyl phosphatidylcholine, **DMPE** dimyristoylphosphatidylethanolamine, **DOGS-NTA** 1,2-dioleoyl-sn-glycero-3-{[N-(5-amino-1-carboxypentyl)-iminodiacetic acid]succinyl}(nickel salt, **DOPE** dioleoyl phosphatidylethanolamine, **DPPA** dipalmitoyl phosphatidic acid, **DPPC** dipalmitoyl phosphatidylcholine, **DPPE** dipalmitoyl phosphatidylethanolamine, **DSPC** distearoyl phosphatidylcholine, **DSPE** distearoyl phosphatidylethanolamine, **DSPG** distearoyl phosphatidyl-glycerol, **DXM** dexamethasone -21 acetate, **DXP** dexamethasone disodium phosphate, **e.c.** epicutaneous, **EL** egg lecithin, **EPC** egg phosphatidylcholine, **EPR** enhanced permeability and retention, **EULAR** european league against rheumatism, **FudR-dP** dipalmitate-5'-fluorodeoxyuridine, **GC** glucocorticoid, **HA** hyaluronan, **HSPC** hydrogentated soybean phosphatidylcholine, **i.a.** intra-articular, **IL** interleukine, **i.p.** intraperitoneaal, **i.v.** intraveneous, **Lf** Lactoferrin, **LUV** large unilamellar vesicle, **mAb** monoclonal antibody, **MLV** large multilamellar vesicle, **MMP** matrix metalloproteinase, **MPS** mononuclear phagocyte system, **MPHS** methylprednisolone hemisuccinate, **MTX** methotrexate, **MTX-DMPE** methotrexate-gamma-dimyristoylphosphatidylethanolamine, **NSAID** non-steroidal anti-inflammatory drug, **OA** osteoarthritis, **OLV** oligolamellar vesicles, **PA** phosphatidic acid, **PAA** poly(acrylic acid), **PC** phosphatidyl choline, **PEG** poly(ethylene glycol), **PEG-S** poly(ethylene glycol) MS400 stearate, **PHEA** poly(hydroxyethyl L-asparagine), **PLP** prednisolone disodium phosphate, **PLP-PEG-L** PLP encapsulated in PEG-liposomes, **POPC** 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, **PS** phosphatidyl serine, **PVA** poly (vinyl alcohol), **RA** rheumatoid arthritis, **ROS** reactive oxygen species, **SA** stearylamine, **SCW** streptococcal cell wall, **SDS** sodium dodecyl sulphate, **siRNA** small interfering RNA, **SLX(-L)** Sialyl-Lewis X (coated liposomes), **SM** porcine brain sphingomyelin, **SOD** superoxide dismutase, **SOD-PEG-L** SOD encapsulated in PEG-liposomes, **SPC** soybean phosphatidylcholine, **SUR I** surfactant I (polyglyceryl-3-cethyl ether), **SUV** small unilamellar vesicles, **TAC** triamcinolone acetonide, **TAC-P** triamcinolone acetonide-21-palmitate, **Tf(s)** Transferosome(s)®, **TNFα** tumor necrosis factor alpha, **TRX-20** 3,5-dipentadecyloxybenzamidine hydrochloride, **VECs** vascular endothelial cells.

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Chapter 2

Pharmaceutical optimization of the liposomal formulation

Chapter 2.1

Optimizing the therapeutic index of liposomal glucocorticoids in experimental arthritis

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Abstract

Small-sized (less than 150 nm) long-circulating liposomes (LCL) may be useful as drug-targeting vehicles for anti-inflammatory agents in arthritis, since they selectively home at inflamed joints after i.v. administration. Previously it was shown in experimental arthritis that encapsulation of glucocorticoids (GC) as water-soluble phosphate esters in PEG-liposomes resulted in a strong improvement of the anti-inflammatory effect as compared to the free drug. In the present study, we compared the therapeutic activity and adverse effects induced by 3 different GC encapsulated in LCL in an attempt to further optimize the therapeutic index of liposomal GC in arthritis. Our data showed that with GC (dexamethasone, budesonide) of higher potency than prednisolone, the therapeutic activity of liposomal GC can be increased. However, side effects at the level of body weight and hyperglycemia were noted, related to the sustained free GC level observed after injection of the liposomal GC. An inverse relationship with the clearance rate of the free GC in question was shown. This study stresses the importance of a high clearance rate of the GC to be encapsulated for achieving a maximal therapeutic index with liposomal GC. Therefore high-clearance GC, which until now are only applied in local treatment approaches, may be very useful for the development of novel, highly effective anti-inflammatory preparations for systemic treatment of inflammatory disorders.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, involving joint inflammation and progressive cartilage destruction (1). Glucocorticoids (GC) are highly effective anti-inflammatory drugs but their use in arthritis therapy is controversial due to a high incidence of serious adverse effects occurring during chronic treatment (2-4). As a result of rapid elimination from the circulation and unfavorable tissue distribution, systemic treatment with GC results in poor target localization of the drug, which often necessitates the use of high doses and intensive dosing schedules (5,6). Targeted delivery of GC can greatly increase the concentration of the drug in the inflamed joints and therefore a less intensive dosing regimen may be sufficient for an adequate therapeutic response with minimal risk for side effects (7).

Long-circulating liposomes have been extensively studied as targeted drug carrier systems in oncology and infectious diseases. Small-sized PEGylated liposomes (less than 150 nm) have been shown to selectively accumulate at the corresponding sites of pathology (8-11). The phenomenon of selective targeting to pathological sites can be attributed to locally enhanced permeability of the vascular endothelium, allowing small-sized PEG-liposomes to extravasate and accumulate in the extravascular tissue (12,13). Larger sized liposomes , on the other hand, have a reduced circulatory half life and accumulate to a higher extend particularly in the spleen, at levels that can trigger high-dose glucocorticoid effects like inhibition of arachidonic acid release and alternative macrophage polarization to the M2 type. They also result in a lower degree of localization in the inflamed joints (14,15).

Previous studies have shown that a single i.v. injection of GC in small-sized long-circulating liposomes yields a rapid, complete and durable disease remission in a rat model of adjuvant arthritis (AA). Prednisolone was selected as model GC and the water-soluble inactive phosphate ester prednisolone phosphate (PLP) was encapsulated to achieve a stable liposome formulation. In its free form, PLP is quickly converted into active prednisolone in blood or tissues. Intensive treatment with repeated daily injections of free PLP could by far not match the effect of a single dose of the liposomal drug. Pharmacokinetic analysis of blood samples taken after administration of liposome-encapsulated PLP revealed not only the presence of liposome-bound PLP but also a low, sustained level of unencapsulated prednisolone in the circulation. The low, sustained level of free prednisolone did not contribute to the therapeutic activity of liposomal PLP (14).

A drawback for the translation of therapeutic effects observed in rat models to humans is that the pharmacokinetic behavior of prednisolone in rats is strongly different from that in human beings (16-18). Therefore, in the present study, we selected dexamethasone phosphate (DXP) for incorporation in liposomes, as the pharmacokinetics of dexamethasone in rats are more comparable to its pharmacokinetics in humans (19,20).

First, we investigated in the AA model whether incorporation of DXP in small-sized poly(ethylene glycol) (PEG) liposomes is also therapeutically beneficial. In view of the stronger potency of dexamethasone over prednisolone higher therapeutic efficacy was anticipated. In previous studies liposomal DXP was shown to be five times more effective compared to liposomal PLP, but it was associated with increased systemic adverse effects, likely due to higher sustained free drug levels (7).

Second, it was evaluated whether the sustained free drug level observed after i.v. administration of liposomal GC is related to the occurrence of systemic side effects. As parameters for systemic side effects, treatment-induced loss of body weight and increase of blood glucose concentration were measured as these parameters can be quickly, frequently and accurately measured (as shown by Kaur et al. (21) and Ogawa et al. (22), respectively).

It was investigated whether the use of a high clearance GC could improve the therapeutic index, by reduction of the occurrence of side effects. The sustained free drug level correlated with the clearance rate of the free GC that appears in the circulation after injection of the corresponding liposomal formulation. The clearance rate is an important determinant of the free drug level, and therefore the chance of systemic side effects may be minimized by encapsulation of a GC with a high-clearance rate. Budesonide sodium phosphate (BUP) was selected as GC with a high clearance rate in this study. BUP has already shown to be more effective than liposomal PLP and DXP in a liposomal formulation in a melanoma B16 murine tumor model, without the occurrence of more adverse effects (23,24). In this study we compared in the AA model small-sized PEG-liposomes containing DXP (relatively slow clearance rate) to small-sized PEG-liposomes containing GC with a higher clearance rate but a lower potency (PLP), and a GC with a higher clearance rate and a higher potency (BUP), with respect to pharmacokinetics, therapeutic response, and systemic side effects, such as body weight loss and increased blood glucose values.

Materials and methods

Preparation of liposomal GC

Small/sized long-circulating PEG-liposomes were prepared by the film-extrusion method (25). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), cholesterol (Sigma Chemical Co., Poole, UK) and distearoyl phosphatidylethanolamine-PEG 2000 (PEG-DSPE) (Avanti Polar Lipids, Alabaster, AL, USA) in a molar ratio of 1.85 : 1.0 : 0.15 respectively. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml of prednisolone disodium phosphate (PLP), dexamethasone disodium phosphate (DXP) (both obtained from Bufa, Uitgeest, The Netherlands) or budesonide disodium phosphate (BUP) (synthesized by Syncom, Groningen, The Netherlands) dissolved in sterile water. The resulting lipid

dispersion was sized to a diameter between 90 and 100 nm by multiple extrusions through polycarbonate filter membranes. The unencapsulated GC-phosphate was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). The mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). The phospholipid content was determined with a phosphate assay (26) in the organic phase after extraction of liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the GC-phosphate content by high performance liquid chromatography, using an isocratic system with a mobile phase of acetonitril-water with a pH of 2, followed by UV-detection at 254 nm. The liposomal preparations contained between 3.5 and 4.5 mg GC-phosphate and an average of 60 µmol phospholipid/ml.

Rat adjuvant arthritis

Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, the Netherlands. To induce arthritis, 100 µl of incomplete Freund's adjuvant (IFA) containing 10 mg/ml of heat-inactivated Mycobacterium tuberculosis (Mt) (both purchased from DIFCO laboratories, Detroit, MI, USA) was injected intracutaneously at the base of the tail (27). At day 10 after the immunization, the first signs of joint inflammation became visible, together with a loss of body weight as a result of the disease. 20 days post-immunization the disease reached maximal severity, after which the inflammation process gradually resolved. Starting at day 10, the rats were daily examined for the visual signs of inflammation and the disease-induced weight drop. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, swelling and deformation of the joints (27). The sum of these four grades for each animal is the clinical score and can vary from zero up to 16. Besides the development of paw inflammation, the disease results in a loss of body weight that could easily be monitored by daily weighing of the rats. The Dutch Committee of Animal Experiments approved all animal studies.

Therapeutic activity

All rats were treated on day 14 or 15 post-immunization, when the average score of all rats in the experiment reached 7, which was approximately half the maximal scores reached in these experiments. At the day of treatment, groups of five rats were formed with equal average clinical scores. All preparations were given intravenously in the tail vein. As the pharmacokinetics of PEG-liposomes have been shown to be lipid dose-independent, the administered dose of phospholipid was allowed to vary with the different liposomal GC preparations (28). When daily injections of free GC were given, each following day treatment was repeated at the same time. The effect of treatment on clinical scores and body weight

loss was monitored daily from day 10 until day 30 post-immunization. Control rats were treated with 150 μmol total lipid/kg empty PEG-liposomes.

Systemic adverse effects

As parameters for systemic activity loss of body weight and increase in blood glucose concentrations were evaluated. Loss of body weight is a phenomenon that is generally observed upon systemic GC treatment in rats (21). In this study the loss of body weight because of treatment with GC was clearly additional to the weight loss resulting from the induction of experimental arthritis. Besides the induction of body weight loss, systemic GC treatment can induce hyperglycemia (22). Monitoring the increase of blood glucose was performed by using a blood glucose meter (EuroFlash, LifeScan Inc, Miltiplas, USA).

Determination of liposomal GC-phosphate and free GC in the circulation

Previously it was shown that PLP remains stably entrapped in PEG-liposomes upon i.v. injection, since at different time-points post-injection PLP was detected in the same quantities as a liposome bilayer marker (assuming that unencapsulated PLP is quickly and completely converted to prednisolone after entering the circulation). Complete retention in the liposomes may also be expected with both DXP and BUP in PEG-liposomes. Despite this, very low levels of free GC in the plasma were observed with liposomal PLP, though these levels were found to not significantly contribute to the increased therapeutic effect of liposomal PLP (14). However, although not evaluated so far, they may contribute to the induction of systemic adverse effects. To evaluate this, plasma concentration-time curves of the different liposomal GC-phosphates were measured after injection of a dose of 10 mg/kg in healthy rats and were compared to PLP-PEG-liposomes. Concentrations of liposomal GC-phosphates were determined by plasma extraction followed by HPLC-determination (29). Concentrations as low as 200 ng/ml could be measured accurately. Quantities of free GC after injection of 10 mg/kg liposomal GC-phosphate in healthy rats could simultaneously be detected with the same assay in a single run with the phosphate ester. Concentrations of free GC in the extracts could be determined accurately down to a concentration of 50 ng/ml.

Statistical analysis

For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding other parameters, a one-way analysis of variance or a Students T-test was performed. P-values of less than 0.05 were considered significant.

Results

Therapeutic activity in rat AA: DXP-PEG-liposomes vs. free DXP

Figure 1 shows the anti-inflammatory effect of 2 mg/kg dexamethasone phosphate (DXP) i.v. encapsulated in PEG-liposomes and in free form. A single dose of 2 mg/kg free DXP significantly suppressed paw inflammation during three days. The same dose encapsulated in PEG-liposomes resulted in complete disappearance of the clinical signs of AA within two days. Complete remission of the disease symptoms lasted until day 20 (6 days post-treatment) after which joint inflammation gradually reappeared, reaching the inflammation score of the saline control group around day 24. The same therapeutic response could be realized by 5 daily injections of 2 mg/kg free DXP.

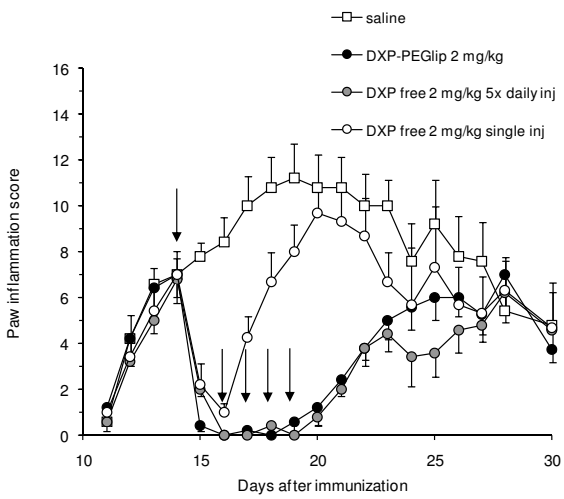

Figure 1: Therapeutic activity in rat AA of 2 mg/kg DXP-PEG-liposomes versus 2 mg/kg free DXP given as single or multiple daily treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates first day of treatment.

Adverse effects: DXP-PEG-liposomes vs. free DXP

Figure 2A shows the effect of the treatment on the total body weight of AA rats. Both liposomal DXP as well as multiple and single treatment with free DXP resulted in treatment-induced weight loss additional to the body weight loss as a result of the disease (saline treatment). Although the same effect was reached at the therapeutic level, repeated administration of 2 mg/kg free DXP generated a stronger treatment-induced loss of body weight than a single injection of 2 mg/kg liposomal DXP ($p < 0.05$ at day 18 post-immunization). In Figure 2B it is shown that 2 mg/kg DXP in liposomal and in free form (single and repeated injections) enhanced blood glucose levels to a similar extent during the first days after treatment.

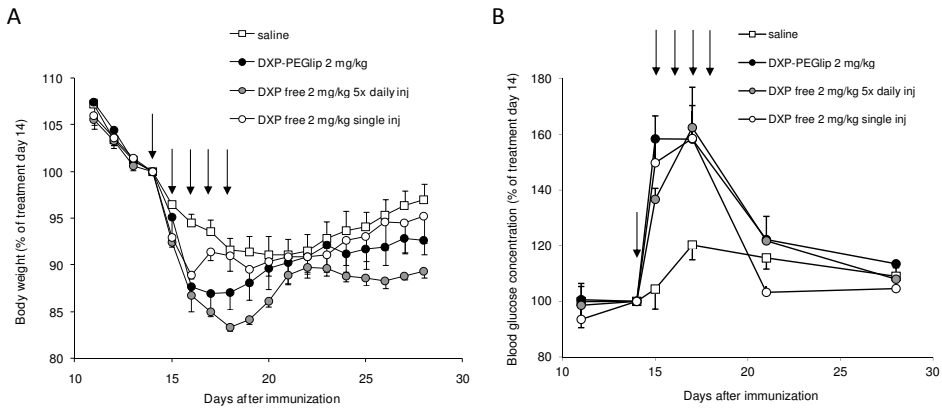

Figure 2: Systemic effects as a result of 2 mg/kg unencapsulated DXP (single and multiple dose), and 2 mg/kg DXP-PEG-liposomes. (A) Effect on total body weight. Vertical bars show SEM. (B) GC-induced hyperglycemia. The percentage of the blood glucose concentration at day of treatment is shown. Vertical bars show SEM. In both A. and B. means of 5 rats are shown. Arrows indicate treatment days.

Therapeutic activity and adverse effects: PLP-PEG-liposomes vs. DXP-PEG-liposomes

To investigate the role of clearance rate of the free GC in the therapeutic index of liposomal GC, prednisolone phosphate (PLP) was encapsulated. However, as these two GC are known to differ in potency, first the therapeutic activities of liposomal PLP and liposomal DXP were compared. Figure 3A shows the comparative effect of a single dose of 2 mg/kg and 10 mg/kg of PLP-PEG-liposomes and DXP-PEG-liposomes on rat adjuvant arthritis scores. There seems to be a correlation between the administered liposomal dose and the resulting therapeutic effect for both liposomal GCs. A dose of 10 mg/kg PLP-PEG-liposomes was equally effective as 2 mg/kg DXP-PEG-liposomes, indicating that liposomal PLP is approximately 5 times less potent than liposomal DXP.

Figure 3B shows that 10 mg/kg PLP-PEG-liposomes reversed the disease-induced process of body weight loss between day 2 and 7 post-treatment. A dose of 2 mg/kg DXP-PEG-liposomes produced a similar response, however, body weight gain started 2 days later, between day 19 and day 24. In the period between day of treatment (day 15) and body weight gain, an additional loss of body weight was observed with liposomal DXP, which was not significant with liposomal PLP. Rats treated with 2 mg/kg liposomal DXP showed an additional weight loss of up to 5.5% as compared to rats in the control group over a period of 4 days before body weight gain was observed. With 10 mg/kg liposomal DXP, this additional body weight loss even reached 9.2%, lasting for more than a week.

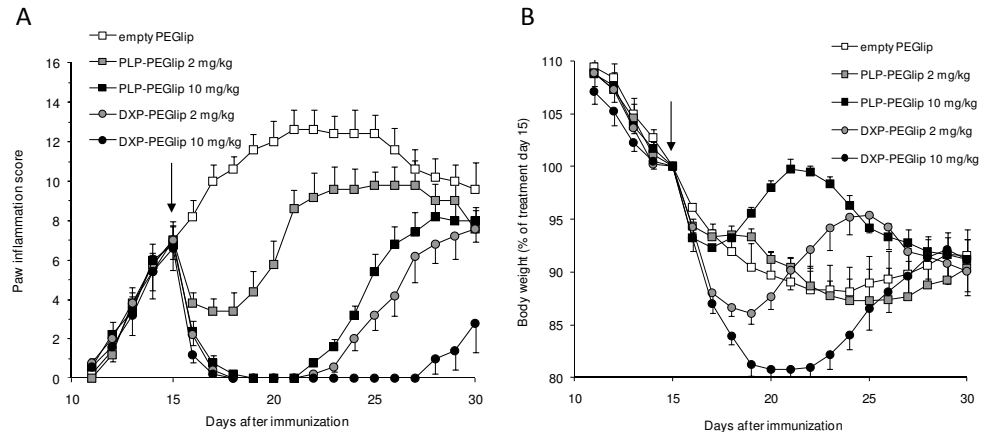

Figure 3: Relative potency of PLP-PEG-liposomes vs. DXP-PEG-liposomes. (A) Effect on joint inflammation in rat adjuvant arthritis, and (B) effect on body weight. Body weight is shown as percentage of the body weight at day of treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates treatment (day 15).

Therapeutic activity and adverse effects: BUP-PEG-liposomes vs. DXP-PEG-liposomes

BUP is known to have a higher clearance rate, compared to DXP, and a higher potency, compared to PLP, and could therefore improve the efficacy and therapeutic index of the liposomal GC even further. The anti-inflammatory effect of a dose of 1 mg/kg BUP-PEG-liposomes was compared to the effect of 1 mg/kg and 2 mg/kg DXP-PEG-liposomes in the rat AA model (Figure 4). Both liposomal DXP and liposomal BUP were highly effective in AA,

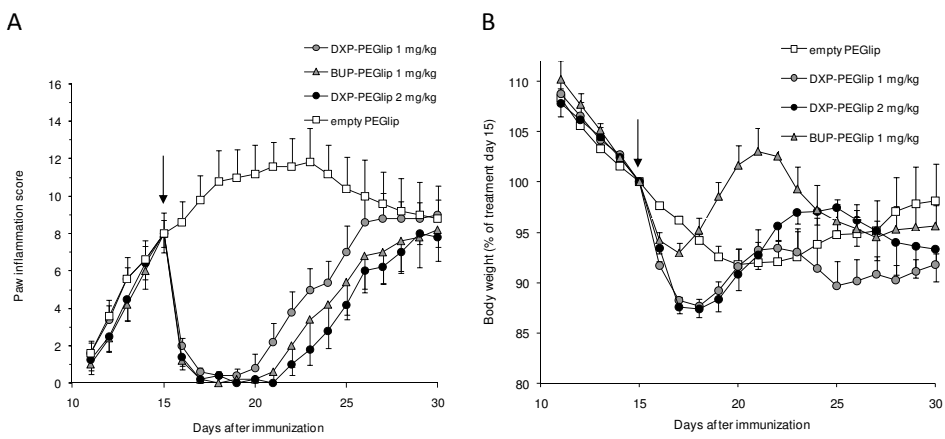

Figure 4: Relative potency and systemic activity of BUP-PEG-liposomes vs. DXP-PEG-liposomes. (A)Effect on joint inflammation, and (B) effect on body weight of 1 mg/kg liposomal BUP and 1 and 2 mg/kg liposomal DXP. Body weight is shown as percentage of the body weight at day of treatment. Means of 5 rats are shown. Vertical bars show SEM. Arrow indicates treatment (day 15)

causing a complete remission of joint inflammation. Liposomal BUP at a dose of 1 mg/kg is equally effective as liposomal PLP at a 10 fold higher dose (Figures 3A and 4A). Importantly, Figure 4B shows that 1 mg/kg liposomal BUP induced an almost complete regain of the disease-induced loss of body weight as a result of its therapeutic effect. This was also seen for liposomal PLP at a dose of 10 mg/kg (Figure 3B). However, the opposite is observed after both 1 mg/kg and 2 mg/kg liposomal DXP, which induced an additional treatment-induced body weight loss. The reversal of disease-induced body weight loss as a result of the therapeutic effect was also seen with liposomal DXP, but this was occurring after the period of additional treatment-induced body weight loss.

Plasma concentrations: liposomal GC vs. free GC

Figure 5A shows the plasma concentration-time profile of the three different GC-phosphates: PLP, DXP and BUP, after injection of a dose of 10 mg/kg encapsulated in PEG-liposomes. All three liposomal GC followed the same plasma concentration-time profile.

Despite equal dose and identical plasma concentration-time profile of the three liposomal GC-phosphates, strong differences were observed regarding the plasma concentration-time profile of the free (i.e. not bound to liposomes) parent drug detected in the circulation after treatment with the liposomal formulations (Figure 5B). Treatment with liposomal DXP yielded the highest free drug levels, whereas treatment with liposomal BUP and PLP resulted in similar, but much lower levels of free GC. Roughly, the areas under the plasma concentration-time curves of free GC in the circulation were inversely correlated with the reported clearance values (in rats: approx. 0.2 $L{\cdot}h^{-1}kg^{-1}$ for dexamethasone (19), 1.5 $L{\cdot}h^{-1}kg^{-1}$ for budesonide (30) and 2.3 $L{\cdot}h^{-1}kg^{-1}$ for prednisolone (16)).

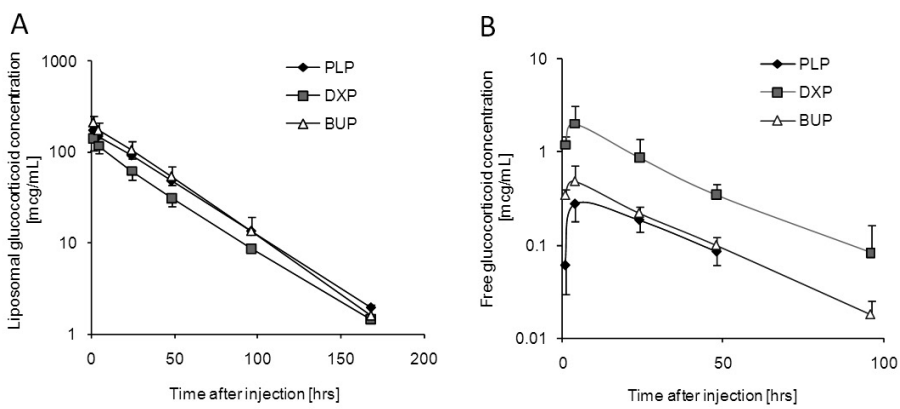

Figure 5: Plasma concentrations of liposomal glucocorticoid phosphate (A) and released free glucocorticoid (B) in the circulation upon injection of 10 mg/kg glucocorticoid phosphate-PEG-liposomes. Data represent means of 4 rats, Vertical bars show SD.

Discussion

In previous studies it was shown that small-sized long-circulating PEG-liposomes extravasate into inflamed joints in experimental rat and murine models of arthritis. Liposomal encapsulation of PLP enhanced the local anti-inflammatory activity to such an extent that a single i.v. injection of 10 mg/kg liposomal PLP was sufficient to induce complete, rapid and long-lasting remission of joint-inflammation. In the present study, we compared the therapeutic activity and adverse effects of 3 different GC, encapsulated in water-soluble phosphate form in small sized long-circulating liposomes in an attempt to further optimize the efficacy of liposomal GC in arthritis. However, increased efficacy is only clinically relevant when the adverse effects are not increased such that the therapeutic index of the drug is not improved upon liposomal encapsulation.

Dexamethasone was chosen as model GC, as its pharmacokinetics in rats are quite similar to that in humans. The first objective was to find the dose at which liposomal DXP induced a therapeutic response comparable to liposomal PLP at a dose of 10 mg/kg. It appears that a single i.v. injection of only 2 mg/kg DXP in small-sized PEG-liposomes can induce a full disease remission for almost a week, indicating that liposomal DXP is indeed approximately 5 times more potent than liposomal PLP (31). Therapeutic benefit could also be realized with free DXP. However, 5 daily injections of 2 mg/kg were required to produce the same response as a single treatment with 2 mg/kg liposomal DXP, indicating that liposomal encapsulation strongly enhances the therapeutic activity of the drug (Figure 1).

The second objective of this study was to evaluate possible systemic side effects induced by the sustained level of free GC in the circulation after injection of liposomal GC. First, the effect on body weight was evaluated. Besides paw inflammation, induction of AA in rats generally leads to a gradual fall of body weight. Therapeutic activity in the model is not only detectable by reversal of paw inflammation, but also by reversal of disease-induced body weight fall (27). Reversal of body weight fall was clearly observed in a previous study with PLP-PEG-liposomes. In the present study, however, instead of a reversal, liposomal DXP induced an extra drop in body weight occurring during the first five days after treatment (Figure 2A). This treatment-induced body weight loss was additional to the disease-induced body weight drop and could be reproduced in healthy rats (data not shown). Body weight loss as a result of i.v. GC has been earlier reported for rats (21,32) and can be considered as a relevant parameter for systemic adverse events. As in our study the treatment-induced body weight fall was also seen in the case of administration of free DXP, it could be that liposomal DXP induced this adverse effect as a result of the presence of free DXP in the circulation.

Besides the effect on body weight also the effect on blood glucose levels of GC can be used as a parameter for systemic activity (22,32). In this study, monitoring blood glucose levels during the course of the disease showed that both liposomal DXP and free DXP caused a limited, but significant hyperglycemia during the first days after treatment (Figure 2B). This

observation again points to the presence of free dexamethasone in the systemic circulation after injection of liposomal DXP. Interestingly, an equipotent dose of liposomal PLP did not result in significant systemic adverse effects. Instead of a treatment-induced body weight loss, a strong regain of body weight was revealed in the first week after treatment with 10 mg/kg liposomal PLP, which clearly corresponded with the remission of paw inflammation (Figure 3). Furthermore, no significant rise of blood glucose concentration was revealed upon 10 mg/kg liposomal PLP (data not shown). These observations suggest that the fraction of the i.v. administered dose of liposomal GC-phosphate that becomes available in the circulation as free GC may be much lower with liposomal PLP than with liposomal DXP due to the higher clearance rate of PLP, or that DXP has a higher intrinsic toxicity compared to PLP.

To investigate this, the third objective of the study was to evaluate whether there is a relation between the clearance rate of the encapsulated GC-phosphate ester and the quantity of free parent drug that becomes available in the circulation after i.v. administration of liposome-encapsulated GC-phosphate. With a higher clearance rate of the circulating free drug, one would expect the quantity of GC present in the circulation in free form to be less. In rats, there is a clear difference between prednisolone and dexamethasone regarding their clearance rate. Our results showed that liposomal encapsulation of PLP results in a stronger improvement of the therapeutic index than liposomal encapsulation of DXP. The absence of systemic activity of liposomal PLP regarding body weight loss (Figure 3B) and hyperglycemia (data not shown) may indeed be explained by the high clearance rate of free PLP in rats. However, this observation may only apply to the rat. In humans, the clearance rate of prednisolone from the circulation is quite similar to that of dexamethasone. To optimize the therapeutic index of i.v. liposomal GC in RA patients, other GC should be selected with high clearance rates after i.v. administration in humans without forming active metabolites. Therefore, we selected BUP. The results show that liposomal BUP is twice as effective as liposomal DXP in rat AA (Figure 4A) while showing less systemic side effect (Figure 4B).

Comparing the plasma concentration-time curves of liposomal BUP with liposomal DXP and liposomal PLP after i.v. injection of equal doses revealed identical profiles for all three liposomal GC. Since it was shown before that no leakage of PLP and DXP from liposomes occurred, such may also be assumed for liposomal BUP. In contrast, the sustained free drug levels after injection of the three liposomal GC formulations greatly differed from each other with an inverse relationship with the clearance rate of the GC in question (Figure 5B). The observation that free budesonide levels were slightly higher than free prednisolone is in agreement with the slightly lower clearance rate of budesonide reported in rats (1.5 $L \cdot h^{-1} kg^{-1}$ as compared to 2.3 $L \cdot h^{-1} kg^{-1}$ for prednisolone) (19,30). However, this does not reflect the human situation, as in humans the clearance rate of prednisolone is much lower than that

of budesonide (17,18), and therefore in the human situation the therapeutic index would be improved even further with liposomal BUP.

In conclusion, our data showed that the use of GC (dexamethasone, budesonide) of higher potency than prednisolone, increases the therapeutic activity of liposomal GC. However, sustained free GC levels were observed after injection of the 3 liposomal GC-phosphates, which showed an inverse relationship with the clearance rate of the GC used. As the sustained free GC levels can cause systemic side effects, this study stresses the importance of a high clearance rate of the free GC in question for achieving a maximal therapeutic index with liposomal GC. Therefore high-clearance GC, which until now are only applied in local treatment approaches, may be very useful for the development of novel, highly effective anti-inflammatory preparations for systemic treatment of inflammatory disorders.

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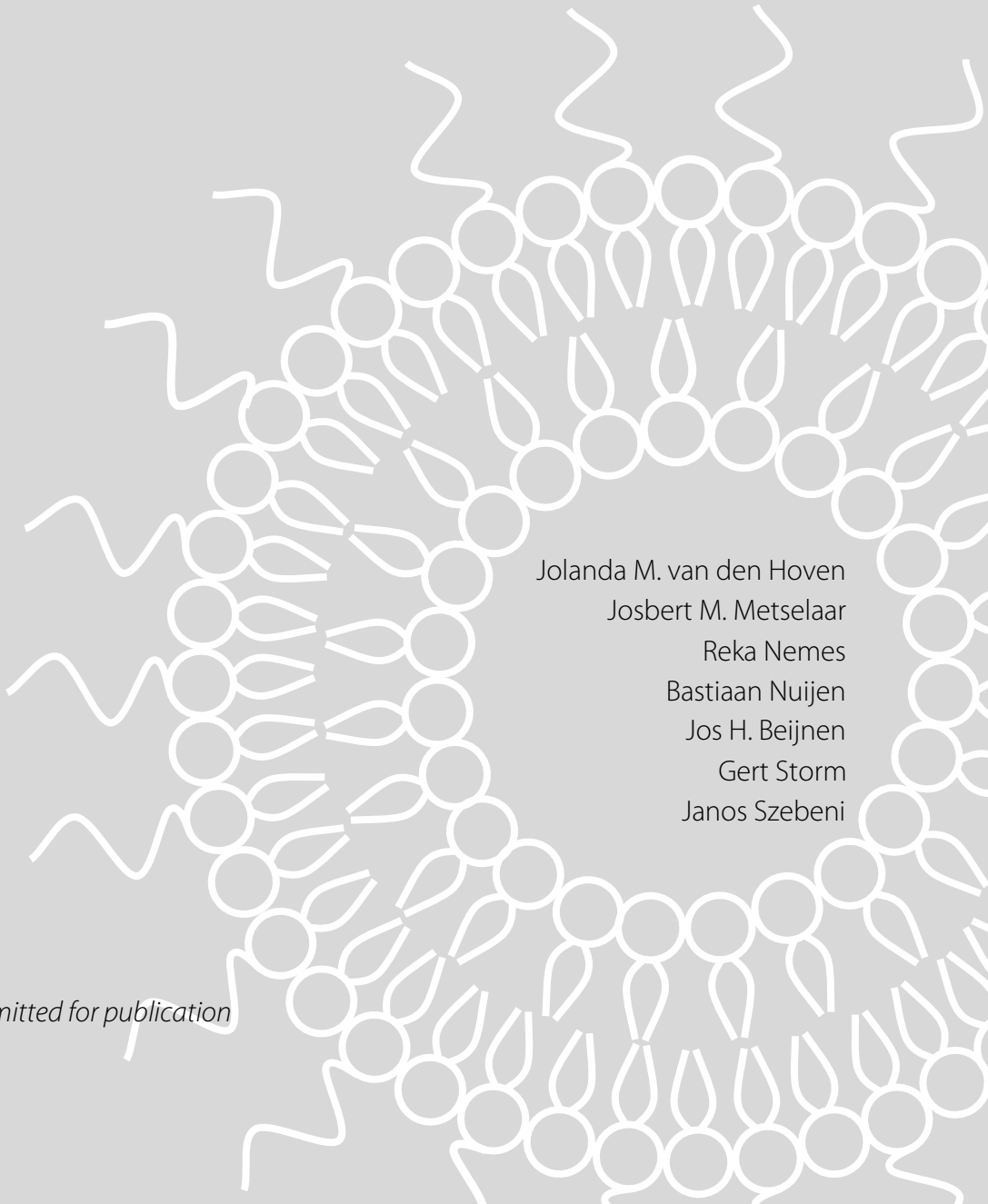
Chapter 2.2

Complement activation by PEGylated liposomes containing prednisolone



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Abstract

Infusion of PEGylated liposomes can give rise to hypersensitivity reactions in a relatively large number of patients. Previously it has been shown that these hypersensitivity reactions can be caused by a negative charge on the anchor molecule of PEG at the liposomal surface. In this study it is tested whether the activation of the complement system by PEG-liposomes could be significantly reduced to values comparable to nonreactive liposomal formulations, by changing the 'PEGylation-profile' on the liposomal surface. Therefore, the formation of complement activation markers SC5b-9, C3a and Bb in normal human serum by both prednisolone loaded and empty liposomes with a variation of PEG chain length, PEG surface concentration, PEG anchor molecule and liposomal size was determined using in vitro assays. It was found that all tested PEGylated liposomal formulations cause mild activation of the complement system. Although this mild activation is not considered as a major risk for hypersensitivity reactions, an occasional reaction cannot be excluded. On the other hand, one particular formulation, wherein the PEG is anchored to cholesterol, turned out to be an extremely strong activator of the complement cascade. This study concludes that PEG anchoring to cholesterol has a major effect on the formation of complement activation markers in vitro, and could therefore be disadvantageous for clinical application of these liposomes. Further study of this phenomenon might be useful for the elucidation of the mechanism of complement activation by PEGylated liposomes.

Introduction

Liposomes have been investigated extensively as drug delivery vehicles to increase the therapeutic index of the encapsulated drug, and their versatility to accommodate a wide range of therapeutic agents has been demonstrated in numerous preclinical and clinical settings (1). Surface modification with a hydrophilic polymer layer, such as poly-ethylene glycol (PEG), opposes their uptake by cells of the mononuclear phagocyte system (MPS) and therefore results in prolonged circulation times. Such long-circulating PEGylated liposomes have demonstrated passive targeting to tumor tissues and inflamed sites, as the locally enhanced vascular permeability allows these drug carriers to extravasate by virtue of the enhanced permeability and retention (EPR) effect. This is one of the mechanisms that improve the therapeutic index of the encapsulated drug (2-4).

However, infusion of PEGylated liposomes can give rise to hypersensitivity reactions in a relatively large number of patients (numbers of up to 30% have been reported) (5-7). The reaction usually occurs at the start of infusion and includes symptoms like cardiopulmonary distress, hypo- or hypertension, dyspnea, tachypnea, facial edema and pain in the chest and back. Most of these hypersensitivity reactions are transient and mild, but life-threatening reactions also have been documented in hypersensitive patients (5,7-12). Since these reactions occur at the first exposure to the drug (without prior sensitization), they are often referred to as 'pseudoallergy' and (because of the causal or contributing role of complement activation) the phenomenon is called 'complement activation related pseudoallergy' (CARPA) (13,14).

Previously it has been shown that these hypersensitivity reactions can be caused by a negative charge on the phospholipid anchor molecule of PEG at the liposomal surface (15). In an attempt to avoid these hypersensitivity reactions, alternative structures for PEG have been developed, like poly(amino acid)s, poly(glycerol) and poly(acrylamide), though these alternative surface structures appeared also to induce activation of the complement system (16,17). To our knowledge, the only long-circulating liposomal formulation that has shown not to activate the complement system is a very small-sized (<70nm) DSPC:CHOL (2:1) liposomal formulation (6). However, a major disadvantage of these small liposomes is their low internal volume, resulting in a low encapsulation efficiency of water-soluble drugs (17).

In 1998, Bradley et al. reported that increasing both the PEG chain length and the molar PEG concentration at the membrane effectively reduces C1q binding, and thereby reduces complement activation via the classical pathway (18). This to us was an indication that by changing the properties of the liposomal PEG-layer, the chance of infusion reactions can be minimized while preserving the long circulating profile. The properties of the PEG layer can be altered by changing the PEG chain length, the anchor molecule for PEG in the liposomal bilayer and the grafting density of PEG. Since the severity of complement activation is also known to be affected by liposomal size, lamellarity, charge, cholesterol content and the

encapsulated drug (5,18), we also investigated the influence of liposomal size, surface charge, and the presence or absence of encapsulated drug (prednisolone disodium phosphate (PLP)).

Several in vitro experiments were performed, the first being an SC5b-9 enzyme-linked immunosorbent assay (ELISA) assay, the second an C3a ELISA assay and finally a Bb ELISA.

SC5b-9 is the soluble, non-lytic form of the terminal complement complex (TCC), the end product of activation of the whole complement cascade. It is generated by the assembly of complement factors C5-C9 and subsequent binding to the regulatory S protein (19). The amount of SC5b-9 is proportional to the total generated TCC and therefore total complement activation. The complement system is activated via three different routes: the classical, lectin and alternative pathways. Activation of all separate pathways results in activation of the terminal pathway, resulting in formation of SC5b-9 (20).

Under normal conditions, activation of all three complement pathways also results in the formation of C3a from C3, which is also quantified by an ELISA. In the alternative pathway, C3 activation results in the formation of Bb, which is quantified by a separate ELISA method, to allow quantitative assessment of the extent of activation of the alternative pathway by the tested formulations.

Materials and methods

Preparation of liposomes

The liposomes were prepared using a film extrusion method (21). Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-PEG2000) (both from Lipoid GmbH, Ludwigshaven, Germany), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethylene-glycol conjugate-550 (DSPE-PEG550, Avanti Polar Lipids Inc., Alabaster, AL, USA), 1,2-distearoyl-SN-glycero-3-phosphatidylethanolamine (DSPE), cholesterol-polyethyleneglycol conjugate-2000 (CHOL-PEG2000) (both from NOF, Grobbendonk, Belgium) and cholesterol (BUFA, Uitgeest, The Netherlands) were dispersed in ethanol in molar ratios as described in Table 1. A lipid film was created by rotary evaporation at 65^{o}C. The lipid film was hydrated with an aqueous solution containing prednisolone disodiumphosphate (BUFA, Uitgeest, The Netherlands) in a concentration of 139 mg/mL, or phosphate buffered saline (PBS) (B.Braun, Melsungen, Germany) in case of empty liposomes. The resulting coarse dispersion was downsized by multiple extrusion steps through polycarbonate filter membranes with pore sizes of 50 or 100 nm to a final pore size as mentioned in table 1. The size was confirmed by Dynamic Light Scattering (DLS). Subsequently, the unencapsulated prednisolone sodiumphosphate (PLP) was removed by dialysis against PBS using Slide-A-Lyzer dialysis

cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10kD, with repeated changing of the dialysis medium.

All compounds used were of pharmaceutical (Ph. Eur) or highly pure (≥99%) grade and were used without any further purification.

Since it is the lipid bilayer that causes the activation, the concentration of bilayer components was kept constant in the tested formulations. Therefore, the lipid content of the liposomal solutions was determined by HPLC and the liposomal solutions were subsequently diluted with PBS to a final total lipid concentration of 55 mM, as these concentrations resulted in successful in vitro analysis previously (6,22).

Table 1: Composition of the tested formulations (molar lipid ratio).

Formulation	Liposomal Size (nm)	PLP	DPPC	DSPE-PEG2000	DSPE-PEG550	DSPE	CHOL	CHOL-PEG2000
A	80	Yes	1.88	0.15	-	-	1.0	-
B	100	Yes	1.88	0.15	-	-	1.0	-
C	120	Yes	1.88	0.15	-	-	1.0	-
D	80	No	1.88	0.15	-	-	1.0	-
E	100	No	1.88	0.15	-	-	1.0	-
F	100	Yes	1.88	-	0.15	-	1.0	-
G	100	No	1.88	-	0.15	-	1.0	-
H	80	Yes	1.88	-	0.15	-	1.0	-
I	100	No	1.88	0.07	-	0.07	1.0	-
J	100	No	1.88	-	-	0.15	0.85	0.15

Abbreviations: PLP, prednisolone sodium phosphate; DPPC, dipalmitoylphosphatidylcholine; DSPE, 1,2-distearoyl-phosphatidylethanolamine; PEG, polyethyleneglycol; CHOL, cholesterol.

Characterization of the liposomes

DLS

The size and size-distribution (polydispersity index, PDI) of the liposomes were determined by DLS with a Malvern ALV CGS-3 system (Malvern instruments Ltd, Malvern, Worcestershire, United Kingdom) with a scattering angle of 90º at 25ºC. Samples were diluted approximately 150 times using phosphate buffered saline (PBS) (B.Braun, Melsungen, Germany) before measurement.

HPLC

Prednisolone phosphate concentrations were determined by HPLC-UV using an 1100 series HPLC system consisting of a binary pump, Model G1312A, an autosampler Model G1367A and a UV-detector Model G1314A (all from Agilent technologies, Amstelveen, The Netherlands). A Zorbax Eclipse-XDB-C8 analytical column (750 x 4.6mm ID, particle size 5 µm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 x 3mm, Varian, Palo Alto, California, USA) were used. Absorbance was measured at 254 nm. Injection of 10 µL of sample was followed by a linear gradient of 5 to 90% acetonitrile (Biosolve B.V., Amsterdam, The Netherlands) with 10mM ammonium formate (Fluka via Sigma-Aldrich, St. Louis, MO, USA). The pH was set at 3.6 using perchloric acid (Merck, Darmstadt, Germany). The flow rate was 1.0 mL/min. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA,USA).

To determine the amount of (un)encapsulated prednisolone disodium phosphate, an additional dialysis step was performed against PBS using Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10kD. A 2 mL sample of the formulation solution was dialyzed against 600 mL of PBS for at least 8 hours. Both the permeate and the retentate were analyzed on the above mentioned HPLC-UV system.

Lipid concentrations of the separate lipid components were determined by HPLC with evaporative light scattering detection (ELSD) using an 1100 series binary HPLC pump, Model G1312A (Agilent technologies, Amstelveen, The Netherlands), AS 3000 autosampler (Thermo Separation Products, Breda, The Netherlands) and an Alltech Varex MKIII Evaporative Light Scattering Detector (ELSD) (Grace (Alltech), Deerfield, IL, USA). An X-Bridge C18 analytical column (750 x 4.6mm ID, particle size 2.5 µm, Waters corporation, Milford, MA, USA) was used. Injection of 30 µL of sample was followed by a linear gradient of 80 to 100% methanol (Biosolve B.V., Amsterdam, The Netherlands) with 1% triethylamine (Merck, Darmstadt, Germany). The flow rate was 0.4 mL/min. Chromatograms were processed using Chromeleon software.

Prior to HPLC analysis the samples were diluted to a concentration of approximately 1 µg/mL prednisolone phosphate or 3 mg/mL total lipid. Subsequently, an extraction using dichloromethane (Merck, Darmstadt, Germany), sterile water for injections (B.Braun) and methanol (Biosolve) was performed on the lipid-containing samples, to separate the prednisolone phosphate and the lipid compounds.

In vitro complement assays in human serum samples

SC5b-9 ELISA

Whole blood samples of 10 healthy volunteers were collected in 10.0 mL BD Vacutainer® silicon coated glass serum tubes with no additives (BD, Franklin Lakes, NJ USA). Blood samples were allowed to clot at room temperature and subsequently centrifuged at 3000 rpm for 5 minutes to collect serum. Serum samples were aliquoted and stored at -20ºC. Frozen samples were rapidly thawed at 37ºC and kept on ice until use.

Complement activation was assessed by Microvue SC5b-9 Plus ELISA kits (Quidel Co., SanDiego, CA, USA). Serum from healthy volunteers was incubated with the diluted (55mM total lipid) liposomal formulations (4:1) in duplicate for 30 minutes at 37ºC, as this concentration resulted in successful in vitro analysis previously (6,22). After incubation the samples were diluted 20-fold in the "sample diluent" of the kit and 100 µl aliquots from this mixture were applied into the wells of the ELISA plate. The assays were performed according to the manual supplied with the kit. The absorbance was measured using a Wallac 1420 Victor 96-wellplate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. SC5b-9 concentrations were calculated using a linear curve fit.

All formulations were tested in serum of 10 different individuals. In this article, the percentage of increase in complement activation marker formation compared to PBS (0% activation level) are used to quantify the activation of the complement system. Chanan-Kahn *et al.* previously showed high specificity and sensitivity of SC5b-9 as a biomarker of Doxil®-induced hypersensitivity reactions in cancer patients, but only when a more than 2-fold elevation of SC5b-9 levels compared to the baseline SC5b-9 level (0.25 ± 0,12 ug/mL (mean ± SD, n= 50) (23)) was seen. Based on sensitivity and specificity, these data suggest that only more than 2-4-fold (100-300%) increases of SC5b-9 levels can be taken as an elevated risk for the occurrence of hypersensitivity reactions, while a more than 4-fold (or 300%) increase is considered clinically relevant for the occurrence of hypersensitivity reactions (7). Therefore, we considered a 100-300% (2-4-fold) increase of the TCC concentration compared to the PBS baseline to be an increased risk on hypersensitivity reactions. In this line of thought, a more than 300% increase in TCC concentration is considered clinically relevant complement activation, with a high risk for hypersensitivity reactions in the patient. Below 100% and above 30% increase is considered to represent a mild activation of the complement system.

Other complement activation markers

C3a and Bb concentrations were determined by Microvue C3a Plus ELISA kits and Microvue Bb Plus ELISA kits respectively (Quidel Co., SanDiego, CA, USA). Serum samples of the 5 most sensitive individuals (as selected from the SC5b-9 concentrations formed) were incubated

with the diluted liposomal formulations (4:1) for 30 minutes at 37ºC in duplicate. Incubated serum samples were diluted 5000-fold and 20-fold for the C3a and Bb analysis respectively, in the supplied "sample diluents" of the kit and 100 µl aliquots from this mixture were applied into the wells of the ELISA plates. The assays were performed according to the kit manuals. The absorbance was measured using a Wallac 1420 Victor 96-wellplate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. C3a and Bb concentrations were calculated based on the calibration curves.

A schematic representation of the ELISA assay principle is given in Figure 1.

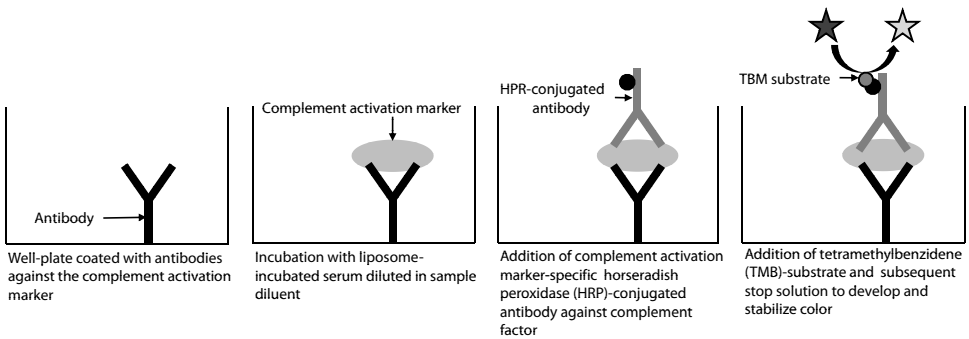

Figure 1: Schematic representation of the ELISA principle.

Results

Characteristics

The liposomal characteristics of the prepared liposomes are given in Table 2. HPLC analysis of the lipid components confirmed the molar lipid ratio of the liposomes as shown in Table 1. Increased PLP concentrations were found upon an increase of the liposomal size, which is a general observation with water-soluble drugs that are (passively) encapsulated in the aqueous interior of the liposome.

In vitro complement activation

First, the formation of the SC5b-9 complex was determined in sera of 10 separate individuals in duplicate. All liposomal formulations induced the formation of SC5b-9 to some extent in a number of sera. Most of these are considered to be mild biologically irrelevant activations, since the TCC concentrations have increased only 30-100% as compared to incubation with PBS, and are not considered statistically significant different ($p>0.05$). However, the formulation in which PEG2000 is coupled to cholesterol instead of DSPE showed a significant ($p<0.05$) 300-1600% increase in TCC concentration in sera of all tested individuals, indicating that this formulation induced extremely strong complement activation (Figure 2,

Table 2: Liposome characteristics (concentrations before dilution to 55mM total lipid concentration)

Formulation	Mean liposomal size (nm)	PDI	Encapsulated PLP (mg/mL)	Total lipid concentration (mM)
A	81	0.06	1.2	55.4
B	97	0.06	3.0	61.5
C	115	0.07	7.4	119
D	82	0.03	-	145.3
E	97	0.17	-	218.2
F	88	<0.01	4.7	98.3
G	94	0.05	-	185.8
H	81	0.01	3.0	111.7
I	95	0.07	-	155.3
J	93	0.03	-	161.2

Abbreviations: PDI, polydispersity index; PLP, prednisolone sodium phosphate.

formulation J). This was confirmed by C3a analysis, in which a substantial increase of up to 1100% of the concentration of C3a was found as compared to PBS incubation. This formulation also is the only formulation that clearly showed significant activation of the alternative pathway, as was reflected by a 100% increase ($p<0.05$) in the concentration of Bb, as compared to incubation with PBS.

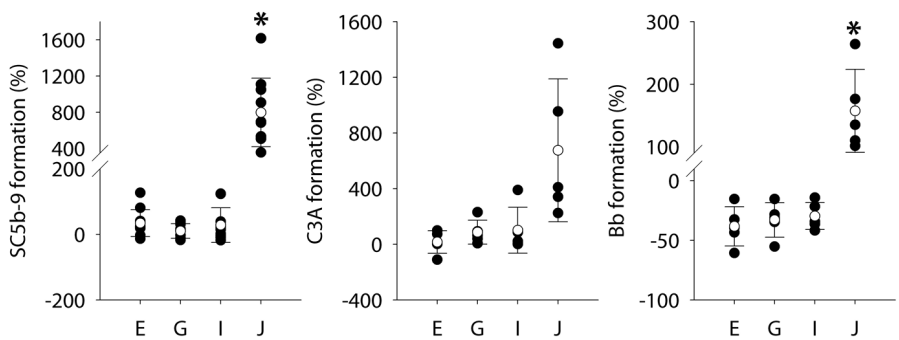

Figure 2: Formation of complement activation markers after incubation of human serum with 100 nm empty liposomes with 5 mol% DSPE-PEG2000 (E), 5 mol% DSPE-PEG550 (G), 2.5mol% DSPE-PEG2000 (I) and 5 mol% CHOL-PEG2000 (J). Exact composition of the liposomes is given in Table 1. Formation of the different complement activation markers is given as a % as compared to incubation with PBS (PBS = 0%). Each black dot represents the mean of a duplicate measurement of the complement activation marker in serum of 1 individual. Empty dots represent the mean of all sera, error bars represent the standard deviation. * = significant complement activation marker formation as compared to the other formulations mentioned ($p<0.05$).

Formulations containing different amounts of DSPE-PEG were compared regarding their ability to activate the complement system. As can be seen from Figure 2, no significant reduction of complement activation was seen when the PEG density at the liposomal surface was reduced. With 2.5 mol% of PEG2000 at the liposomal surface (Figure 2, formulation I) instead of 5 mol% (Figure 2, formulation E), a comparable mean level of SC5b-9 formation, as well as a comparable number of sera showing (mild) increase of C3a formation were found. Indeed, serum of one individual showed >300% increase of C3a formation after incubation with the formulation with the lowered surface concentration of PEG2000 (Figure 2, formulation I), which might be suggestive of a higher risk of hypersensitivity reactions with this formulation.

It was hypothesized that liposomes with a shorter PEG-chain could cause less activation of the complement system. Therefore, formulations were prepared containing PEG550 instead of PEG2000. As can be seen from Figure 2 (formulation E and G). reducing the PEG chain lengths to PEG550 did not result in a significant decrease in formation of SC5b-9 and C3a.

It was expected that an increase in liposomal size could increase complement activation marker formation. However, the mean levels of both C3a and SC5b-9 as well as the number of sera of individuals showing (mild) activation does not significantly change (Figure 3). An approximately 300% increases of C3a formation as compared to PBS was seen in serum of the same individual for both the smallest (80 nm) and the largest size (120 nm) tested (Figure 3, formulation A and C). The same was found when comparing different sizes of PEG550 formulations (Formulation G and H, data not shown). Only the largest liposomal size tested (120 nm) showed a significantly increased activation of Bb (Figure 3), indicating that size might be important for complement activation by triggering the alternative pathway.

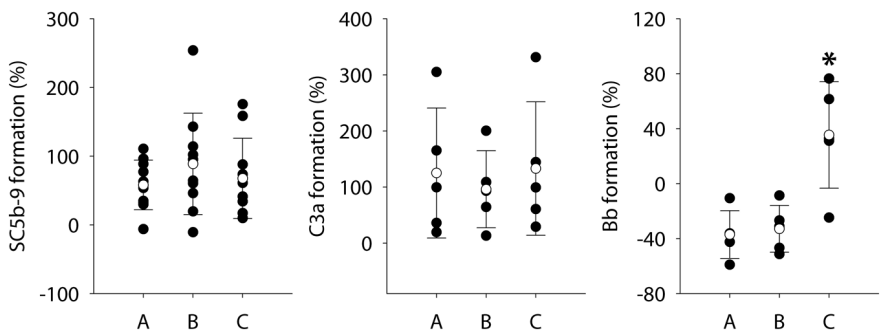

Figure 3: Formation of complement activation markers after incubation with liposomes increasing in size. Human serum was incubated with PLP loaded liposomal formulations containing 5 mol% DSPE-PEG with sizes of approximately 80 nm (A), 100 nm (B) and 120 nm (C). Exact composition of the liposomes is given in Table 1. Formation of the different complement activation markers is given as a % as compared to incubation with PBS (PBS=0%). Each black dot represents the mean of a duplicate measurement of the complement activation marker in serum of 1 individual. Empty dots represent the mean of all sera, error bars represent the standard deviation. *=significant complement activation marker formation as compared to the other formulations mentioned ($p<0.05$).

PEGylated liposomes containing PLP tend to show increased complement activation as compared to their empty liposomal counterparts (Figure 4). However, this increase of mean levels of complement activation markers is only significant for PEG550 liposomes in the Bb assay. For two of the tested formulations, namely 100 nm PEG2000-liposomes with PLP and 100 nm PEG550-liposomes with PLP (formulations A and F respectively), serum of only one individual revealed a >300% increase of C3a levels versus PBS. Overall, this suggests that there might be an inducing effect of the drug in the liposomal formulation at the level of complement activation. This phenomenon has been seen with Doxil® (doxorubicin in PEGylated liposomes), and has been related to doxorubicin indirectly contributing to complement activation by changing the liposomal surface curvature (5,14) and promoting aggregate formation (24).

Discussion

The objective of this study was to investigate if changing the properties of the PEGlayer on the liposomal surface reduces complement activation that has been associated with the hypersensitivity reactions upon infusion of PEGylated liposomes (5-7). Therefore, we measured the concentration of several complement activation markers that were formed in vitro after incubation with liposomal formulations with different PEG layer properties.

Three complement activation markers were selected that are known markers of complement activation in different routes and stages. Specifically, C3a is a measure of C3 activation, Bb reflects activation of the alternative pathway, while SC5b-9 provides a measure of the whole cascade until TCC. The sera of the individuals in this study were first screened for TCC formation since this factor is known to be a sensitive and consistent measure of complement activation in human blood (25,26).

Previous studies have shown that more than 2-4-fold (100-300%) increases of SC5b-9 levels in in vitro complement assays may be considered as an elevated risk for the occurrence of hypersensitivity reactions in vivo, while a more than 4-fold (or 300%) increase is considered a clinically relevant risk for the occurrence of these reactions (7). Since all formulations, except the CHOL-PEG2000 formulation showed only mild mean levels of complement activation (less than 100% increased mean concentrations of SC5b-9 as compared to PBS), our data suggest that the PEGylated liposomal formulations studied here have low risk for causing hypersensitivity reactions in man. However, occasional reactions cannot be excluded, as shown by the higher values of complement activation (exceeding 100% increase in TCC concentrations) in sera of specific individuals in our study.

Extensive research has been published concerning the occurrence of hypersensitivity reactions after administration of (PEGylated) liposomes. Various groups have shown that factors such as multilamellarity, a large size (>200 nm), a non-circular shape, a high

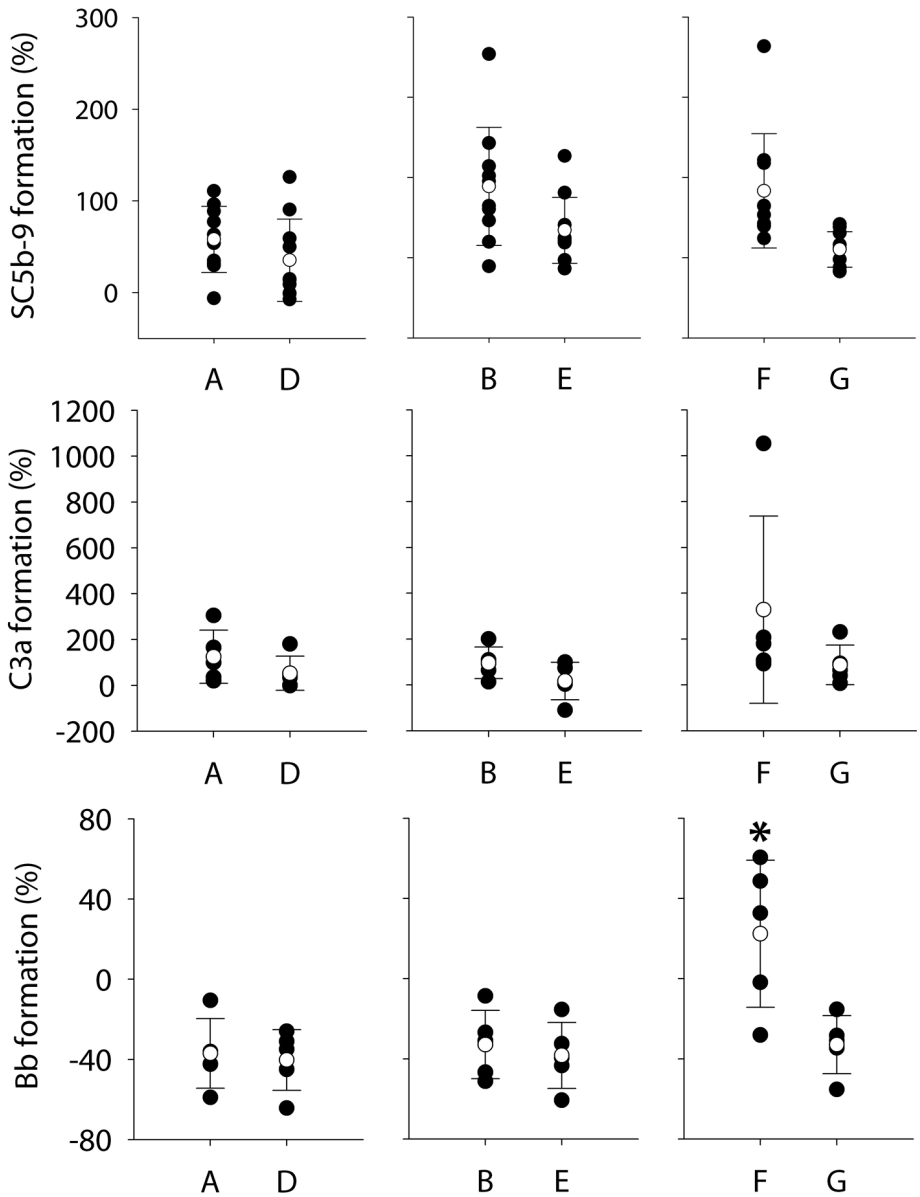

Figure 4: PEGylated liposomes with PLP tends to increase activation of the complement system compared their empty counterparts, as was shown for liposomes containing 5 mol% DSPE-PEG2000 of 80 nm (A with encapsulated PLP and D without PLP) and 100 nm (B with encapsulated PLP and E without PLP) respectively, as well as for 100 nm liposomes containing 5 mol% DSPE-PEG550 (F with encapsulated PLP and G without PLP). Each black dot represents the mean of a duplicate measurement of the complement activation marker in 1 individual. Empty dots represent the mean of all individuals, error bars represent standard deviations. * = significant complement activation marker formation as compared to the equivalent empty formulation ($p<0.05$).

cholesterol content (≥71%) and a high infusion rate (or bolus injection) all significantly increase the chance of complement activation (27,28). Previously it was thought that addition of PEG to the liposomal surface would reduce the interaction with plasma proteins and thus with complement factors (as was shown in vitro for C1Q) in the systemic circulation (18,27). However, PEGylated liposomal products, like Doxil®, have been shown to induce complement activation-related hypersensitivity reactions in patients (5-7,12). This activation has been related to the presence of an anionic phosphate moiety in the PEG-lipid conjugate, though the mechanism of the phenomenon remains elusive (28). An alternative for PEG without this effect has not yet been found, and therefore PEG still remains the golden standard in the formulation of long circulating liposomes (16,17,22). In this study, it was found that a reduction of PEG chain length caused no significant changes in the activation of the complement system in vitro, nor did a reduction of the PEG density at the surface, at least until 2.5 mol%.

Previously it has been shown for Doxil®, that the presence of doxorubicin influences the activation of the complement system (5,6). It was hypothesized that the activation could be caused by a change in physicochemical liposomal surface properties due to the formation of elongated doxorubicin crystals in the liposomal aqueous core. In our study however, there also seems to be a tendency to an increased formation of complement activation markers after loading the PEGylated liposomes with PLP, which is encapsulated as a solution in the liposomal aqueous interior. We cannot exclude that PLP and other drugs that are encapsulated in dissolved form rather than as crystals, can also cause slight changes to the liposomal formulation that result in an increased chance of complement activation. This warrants a more in depth investigation of the physicochemical characteristics of PLP-PEGylated liposomal formulations.

The formulation in which cholesterol was used as the anchor molecule for PEG, instead of normally used DSPE, showed unexpected strong complement activation. Although unsuitable as a potential new drug delivery vehicle in view of the activation results, this formulation might provide a new tool for better understanding the mechanism of complement activation by PEGylated liposomes. CHOL-PEG2000 differs from DSPE-PEG2000 in that the former has no negatively charged phosphate group in its lipophillic anchor that stabilizes the position of the PEG-lipid-conjugate relative to the membrane surface. Although this charged phosphate group appeared to be the main cause of complement activation by PEG-DSPE (15), leaving it out might lead to much more efficient complement activation. This phenomenon of triggering of complement activation is not unprecedented, and has also been encountered in the field of carbon nanotubes (12,29,30). Although highly hypothetical at present, it is conceivable that the hydrodynamic forces on the hydrophilic PEG chain results in a tendency to extract the cholesterol anchor out of the lipid membrane, exposing the antibody-binding portion of cholesterol to natural anti-cholesterol antibodies, which causes an even stronger activation of complement (anti-cholesterol antibodies

appear very strong complement triggers) (31-33). Future studies will have to test the validity of this hypothesis.

In summary, we found that PLP containing PEGylated liposomes can mildly activate the complement system in the majority of normal human serum. This suggests a relatively small risk for infusion reactions. However, an occasional reaction cannot be excluded. One particular formulation, wherein the PEG is conjugated to cholesterol as a lipophillic anchor molecule, turned out to be an extremely strong activator of the complement cascade. Further study of this phenomenon might be useful for the elucidation of the mechanisms behind complement activation by liposomal formulations in general.

Acknowledgements

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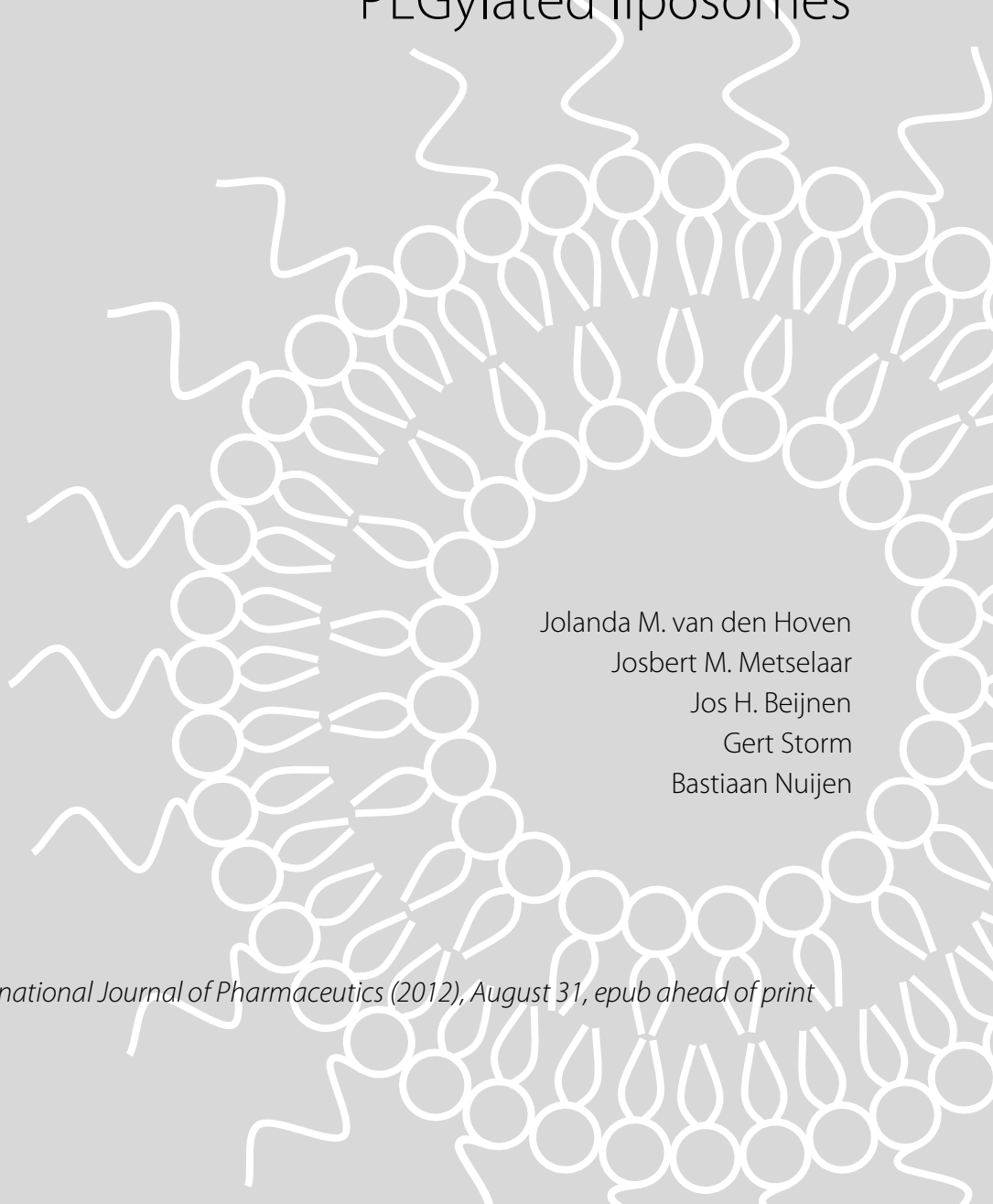
Chapter 2.3

Cyclodextrin as membrane protectant in spray-drying and freeze-drying of PEGylated liposomes



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Abstract

In this study it was investigated whether hydroxypropyl-β-cyclodextrin (HPβCD) is able to stabilize the liposomal membranes during drying of long circulating polyethylene glycol (PEG) coated liposomes, as compared to the disaccharides trehalose and sucrose. PEGylated liposomes loaded with prednisolone disodium phosphate (PLP) were dried by spray-drying or freeze-drying. The dried powders were tested on their residual moisture content, glass transition temperature and amorphous character. Upon reconstitution the liposomal size, size distribution and drug retention were determined and the results were compared to the characteristics of the formulation solution before drying. In contrast to the disaccharides, HPβCD stabilizes the liposomal membranes of the PEGylated liposomes during the drying process of both spray drying and freeze-drying when present in a lipid:carbohydrate ratio of 1:6 (w/w). The resulting powder can be stored at room temperature. No changes in size and size distribution were seen upon reconstitution of the HPβCD containing formulations. Drying resulted in a minimal leaking of PLP from the liposomes. Its relatively high T_g' and T_g of HPβCD, as compared to the disaccharides, make HPβCD an excellent membrane protectant for dry PEGylated liposomal formulations.

Introduction

Liposomes have proven to be well tolerated drug delivery vehicles that offer the possibility of targeted drug delivery for a wide range of therapeutic agents (1). Physicochemical properties of liposomes can be changed to optimize drug delivery and retention at the target site, thus enhancing their therapeutic efficacy, and to prevent toxicity to non-target tissues (2-5). Furthermore, liposomes can offer a solution in case of formulation problems of the active compound as a result of for instance low aqueous solubility (6-8). However, the phospholipids in the liposomal membrane, especially when dispersed in water, can slowly become oxidized or hydrolyzed (9-12). This could induce fusion of liposomes, leakage of the enclosed drug compound, and structural transformations of the liposomes, which might influence their performance (13). Dry products generally show higher stability, and therefore various groups have tried to develop dried liposomal formulations (10,14-22). Apart from a stabilization objective, dry liposomal formulations also offer opportunities for routes of administration other than parenteral use only, e.g. as dry powder inhalation.

Commonly applied drying techniques in pharmaceutical manufacturing are spray drying and freeze-drying. Freeze-drying of conventional liposomes (e.g. liposomes without surface modifications) has been well documented in the literature (9,10,14,19,23). Though less frequently, freeze-drying of long circulating liposomes containing polyethylene glycol (PEG) has also been reported (17,21,24,25) However, as compared to lyophilization, only a limited number of reports have focused on spray-drying as a method to dry liposomal formulations (17,26-29). Most groups tried to spray-dry conventional liposomes, though Wessman et al. investigated the effect of spray-drying on the structure of PEGylated liposomes (17). Compared to freeze-drying, spray-drying is much faster, less expensive and more suited for production of defined particles (30). On the other hand, freeze-drying is more suited for the development of sterile drug products. Spray-drying results in a powder mass that requires subsequent handling into the final product format whereas freeze-drying offers the possibility of drying defined volumes of the aqueous formulation in the final product container.

The main issue in drying of liposomal formulations is the stability of the liposomal membranes. These membranes can be easily disrupted during the drying process, for instance due to ice crystals or phase transition of the membranes under influence of temperature, or due to sublimation of water from the liposomal surface (10,30,31). Therefore, the liposomal membranes needs to be protected during the drying process. Cryo- and lyoprotectants that are often used to protect delicate structures like proteins, DNA and liposomes during drying processes are disaccharides like sucrose and trehalose (19-22,27,32). Disaccharides are able to form hydrogen bonds, thereby stabilizing the ordered conformation of the delicate structures upon removal of water molecules (water replacement theory) (33,34).

Besides disaccharides, hydroxypropyl-β-cyclodextrin (HPβCD), a cyclic oligosaccharide, has also proven to stabilize proteins during spray-drying (35,36). The exact mechanism is still unknown but might be improved vitrification due to a higher vitrification temperature (the glass transition temperature of maximally cryoconcentrated solutions, T_g') and/or improved water replacement due to its large number of hydrogen donors and acceptors (35-39). HPβCD has a high aqueous solubility and a safe toxicity profile for a variety of administration routes, including parenteral use (40-42). Several products containing HPβCD have been marketed, e.g. Sporanox® and Trisporal® (containing itraconazol) and Indocollyre® (containing indometacin) (43).

In this study it was investigated whether HPβCD is able to stabilize the liposomal membranes during both spray-drying and freeze-drying of long circulating PEGylated liposomes, as compared to the disaccharides trehalose and sucrose. The PEGylated liposomes were loaded with the water-soluble drug prednisolone disodium phosphate (PLP) as a model drug. Creating a dry liposomal formulation of a water-soluble drug encapsulated in the aqueous core of the liposome is a major challenge, since the drug can leak out of the liposome during drying (14). Therefore, drug leakage is a good marker for instability or even rupture of the liposomal membranes during the drying process. From our own experience we know that PLP does not leak out of the PEGylated liposomes in aqueous dispersion (Nanocort; (44)). Also, PLP solutions are chemically stable for considerable time. Based on these characteristics, we selected PLP-PEGylated liposomes as a model drug formulation. During drying, the water is removed from both outside and inside the liposomes. Therefore, it might be relevant to protect the liposomal membrane on both sides (22,32). To evaluate this, liposomal formulations were prepared both with and without lyoprotectant present in the liposome core. Besides drug retention, physicochemical properties and microscopic appearance of the dried liposomal formulations were investigated.

Materials and methods

Preparation of the liposomes

Liposomes were prepared using a film extrusion method (45). Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-PEG) (both from Lipoid GmbH, Ludwigshaven, Germany) and cholesterol (BUFA, Uitgeest, The Netherlands) were dissolved in ethanol. A lipid film was created by rotary evaporation at 65°C. The lipid film was hydrated with a solution containing prednisolone disodium phosphate (BUFA, Uitgeest, The Netherlands) in a concentration of 139 mg/mL. Furthermore, the hydrating solutions contained either 0% or 10% of sucrose (BUFA, Uitgeest, The Netherlands), trehalose (Merck, Darmstadt, Germany) or HPβCD (Roquette Pharma, Lestrem, France) in sterile water for injections (B.Braun,

Melsungen, Germany). The resulting coarse dispersion was sized by multiple extrusion steps through polycarbonate filter membranes with a pore size of 100 nm, resulting in liposomes with a diameter of about 100 nm, as was confirmed by dynamic light scattering (DLS). Unencapsulated PLP was removed by dialysis against a 10% solution of sucrose, trehalose or HPβCD using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kDa, with repeated changing of the dialysis medium. The lipid content of the liposomal dispersions was determined using HPLC, and the liposomal dispersions were subsequently diluted with their corresponding 10% sugar solutions to a final ratio of sugar:lipid of 6:1 (w/w, dry product), as ratios of 4:1 or higher have shown to protect the liposomes during drying in previous studies (21,23,46). The diluted dispersion is used for the drying processes.

All compounds used were of pharmaceutical (Ph. Eur) or highly pure (≥99%) grade and were used without any further purification.

Spray-drying of the liposomes

The aqueous formulations were spray-dried using a B-290 Mini Spray Drier (Büchi Labortechnik GmbH, Hendrik-Ido-Ambacht, The Netherlands). The spray-drying conditions were selected based on literature (16,28,29) and were as follows: inlet and outlet temperatures were 100 ºC and 68 ºC, respectively; airflow rate was 35 m^3/h and the spray gas flow was 670 L/h; with a nozzle size of 0.7/1.5 mm; the feed was set at 1 mL/min. The resulting spray-dried powders were kept in closed containers at 2-8 ºC prior to characterization and further analysis.

Freeze-drying of the liposomes

1 mL aliquots of the liposomal dispersions were filled into 8R colorless glass vials (hydrolytic class type 1 Fiolax clear, Aluglas, Uithoorn, The Netherlands). Vials were partly closed using gray bromobutyl rubber lyophilization closures (West Pharmaceutical Services Inc., Lionville, PA, USA) and loaded into the freeze dryer (Model Lyovac GT4, GEA Lyophil GmbH, Hürth, Germany). The lyophilization program was based on the literature (14,47,48). Vials were frozen to -35 ºC at 0.5 ºC/min in two hours. The shelf temperature of -35 °C was maintained for 24 hours during the primary drying phase, while a vacuum of 10 Pa was established. At the end of primary drying the temperature was linearly increased to 0 °C in 2 hours while the pressure was reduced to 0.9 Pa, to start secondary drying. These conditions were maintained for another 48 hours after which the vials were stoppered pneumatically under vacuum, removed from the freeze-dryer, and stored at 2-8 ºC prior to characterization and further analysis.

Characterization of the dried powders

Visual inspection of the powders

The appearance, bulk density and flowability of the dried powder formulations were compared by visual inspection.

Differential scanning calorimetry (DSC)

DSC was performed using a Q2000 DSC equipped with a refrigerated cooling accessory (RCA) for low temperature in the T4P mode (TA instruments, New Castle, DE, USA). Temperature scale and heat flow were calibrated with indium. For determination of the glass transition temperature (T_g) powder samples of approximately 5-10 mg were transferred into TZero Aluminium pans (TA Instruments) and closed hermetically. The sample was equilibrated at 0 °C, followed by an isothermal step for 5 minutes. Subsequently the sample was heated to 80 °C with 2°C/min and 1 °C/60 sec modulation. An empty pan was used as a reference.

For determination of the membrane transition temperature (T_m), samples of approximately 10 mg of the reconstituted powder solutions were transferred into TZero Aluminium Hermetic pans (TA instruments) and closed hermetically. The sample was equilibrated at 10°C, followed by an isothermal step for 5 minutes. Subsequently the sample was heated to 80°C with 5°C/min. An empty pan was used as a reference.

Residual moisture content

Determination of the residual moisture content of the dried product was performed using the Karl Fisher titration method. Approximately 90 mg of the dried formulation was transferred into the titration unit of a Model 658 KF Titrino apparatus (Metrohm, Herisau, Switzerland).

X-ray diffraction

X-ray powder diffraction measurements were performed using an X'pert pro diffractometer equipped with an X-celerator (PANanalytical, Almelo, The Netherlands). A 0.5 mm deep metal sample holder was filled with sample. The particle size of granule-like structures and crystalline materials was reduced using mortar and pestle before filling of the sample holder. Subsequently the sample was placed in the diffractometer. Samples were scanned at a current of 50 mA and a tension of 40 kV. The scanning range was 10–100◦ 2θ, with a step size of 0.020◦ and a scanning speed of 0.002◦ per second.

Characterization of the liposomes

The dried formulations are reconstituted with sterile water for injections (B.Braun) to their original concentrations (w/v). The resulting liposomal solutions were characterized and the results were compared to the characteristics of the formulation solution before drying.

Visual inspection of the formulation

The liposomal solutions were compared by visual inspection. The turbidity, the degree of opalescence and the color of the solution were compared. Additionally, the reconstitution time was determined.

Dynamic light scattering (DLS)

The size and size-distribution (polydispersity index, PDI) of the liposomes was determined by DLS with a Malvern ALV CGS-3 system (Malvern instruments Ltd, Malvern, Worcestershire, United Kingdom) with a scattering angle of 90º at 25 ºC. Samples were diluted approximately 40 times using phosphate buffered saline (PBS) (B.Braun, Melsungen, Germany) before measurement.

High pressure liquid chromatography (HPLC)

PLP concentrations were determined by HPLC-UV using an 1100 series HPLC system consisting of a binary pump, Model G1312A, an autosampler Model G1367A and a UV-detector Model G1314A (all from Agilent Technologies, Amstelveen, The Netherlands). A Zorbax Eclipse-XDB-C8 analytical column (750 x 4.6mm ID, particle size 5 µm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 x 3mm, Varian, Palo Alto, California, USA) were used. Absorbance was measured at 254 nm. Injection of 10 µL of sample was followed by a linear gradient of 5 to 90% acetonitrile (Biosolve B.V., Amsterdam, The Netherlands) with 10mM ammonium formate (Fluka via Sigma-Aldrich, St. Louis, MO, USA). The pH was set at 3.6 using perchloric acid (Merck, Darmstadt, Germany). The flow rate was 1.0 mL/min. Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

To determine the amount of (un)encapsulated PLP, an additional dialysis step was performed against a 10% solution of sucrose, trehalose or HPβCD using Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kDa. A 2 mL sample of the formulation solution was dialyzed against 600 mL of medium for at least 8 hours. Both the permeate and the retentate were analyzed on the above mentioned HPLC-UV system.

Lipid concentrations were determined by HPLC with evaporative light scattering detection (ELSD) using an 1100 series binary HPLC pump, Model G1312A (Agilent Technologies, Amstelveen, The Netherlands), AS 3000 autosampler (Thermo Separation Products, Breda,

The Netherlands) and an Alltech Varex MKIII Evaporative Light Scattering Detector (ELSD) (Grace (Alltech), Deerfield, IL, USA). An X-Bridge C18 analytical column (750 x 4.6mm ID, particle size 2.5 µm, Waters Corporation, Milford, MA, USA) was used. Injection of 30 µL of sample was followed by a linear gradient of 80 to 100% methanol (Biosolve B.V., Amsterdam, The Netherlands) with 1% triethylamine (Merck, Darmstadt, Germany). The flow rate was 0.4 mL/min. Chromatograms were processed using Chromeleon software.

Prior to HPLC analysis the samples were diluted, if necessary, to a concentration of approximately 1 µg/mL PLP or 3 mg/mL total lipid. Subsequently, an extraction using dichloromethane (Merck, Darmstadt, Germany), sterile water for injections (B.Braun) and methanol (Biosolve) was performed on the lipid-containing samples, to separate the PLP and the lipid compounds.

Transmission electron microscopy (TEM)

The size and shape of the liposomes were visualized using TEM. To this end, samples diluted 1000 times were applied on Agar® formvar/carbon coated copper grids (van Loenen instruments, Zaandam, The Netherlands). The samples were negatively stained by uranyl acetate and dried on air. The samples were visualized under a Tecnai12 transmission electron microscope (Philips, Eindhoven, The Netherlands) using a GATAN 626 cryoholder (Gatan GmbH, München, Germany). Samples were observed at 120 kV. Images were recorded on TemCam-0124 camera (TVIPS GmBH, Gauting, Germany) and processed with AnalySIS software. The magnification ranged from 30,000 to 265,000 times.

Table 1: Liposomal characteristics before drying (PDI=polydispersity index). Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar)

Formulation		Size (ø, nm)	PDI	Total lipid (mg/mL)	Molar lipid ratio (DPPC: DSPE-PEG:CHOL)	PLP content (mg/mL)
No internal sugar	Sucrose	100	0.05	13.61	2.4 : 0.14 : 1.0	0.79
	Trehalose	100	0.03	15.73	2.4 : 0.14 : 1.0	1.12
	HPβCD	112	0.04	12.10	2.6 : 0.15 : 1.0	1.54
10% internal sugar	Sucrose	104	0.09	14.22	2.4 : 0.13 : 1.0	0.86
	Trehalose	98	0.10	14.64	2.4 : 0.14 : 1.0	1.32
	HPβCD	104	0.01	13.97	2.6 : 0.16 : 1.0	1.79

Results

Characterization of the liposomes

PLP-PEGylated liposomes were prepared in order to evaluate the stabilizing effect of the different carbohydrates on the liposomal membranes during drying. The liposomal characteristics before drying were determined using HPLC-UV, HPLC-ELSD and DLS. All different liposomal formulations had comparable characteristics before drying (Table 1).

The effect of the drying methods

Visual inspection of the dried liposome formulations showed no differences in powder characteristics between the formulations with and without internal carbohydrate. However, differences were observed between the spray-dried formulations and the freeze-dried formulations. The powder resulting from spray-drying using HPβCD as lyoprotectant consisted of finer particles with a lower bulk density as compared to the disaccharide-containing powders, that consisted of granule-like particles. Lyophilization of the liposomal solutions resulted in white cake structures with a residual water content of approximately 1%, whereas spray-drying resulted in white powders with a residual water content of approximately 4%, irrespective of the formulation (Table 2).

Table 2: Residual moisture content and T_g values of the dried formulation. Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar)

Formulation		Residual moisture content (%)		T_g (°C)	
		After freeze-drying	After spray-drying	After freeze-drying	After spray-drying
No internal sugar	Sucrose	0,97	3,61	39	36
	Trehalose	1,20	3,90	48	50
	HPβCD	1,37	4,70	>100	>100
10% internal sugar	Sucrose	0,97	3,58	37	36
	Trehalose	1,06	4,04	50	50
	HPβCD	1,28	4,26	>100	>100

X-ray diffraction analysis showed characteristic crystalline diffraction peaks in the X-ray diffraction spectra of the unprocessed disaccharides (Figure 1: 1 in panel A and B). These were

absent in the unprocessed HPβCD (Figure 1: 1 in panel C), as well as in all dried formulations, indicating that the dried PLP-PEGylated liposome formulations are amorphous (Figure 1: 2-5 in all panels).

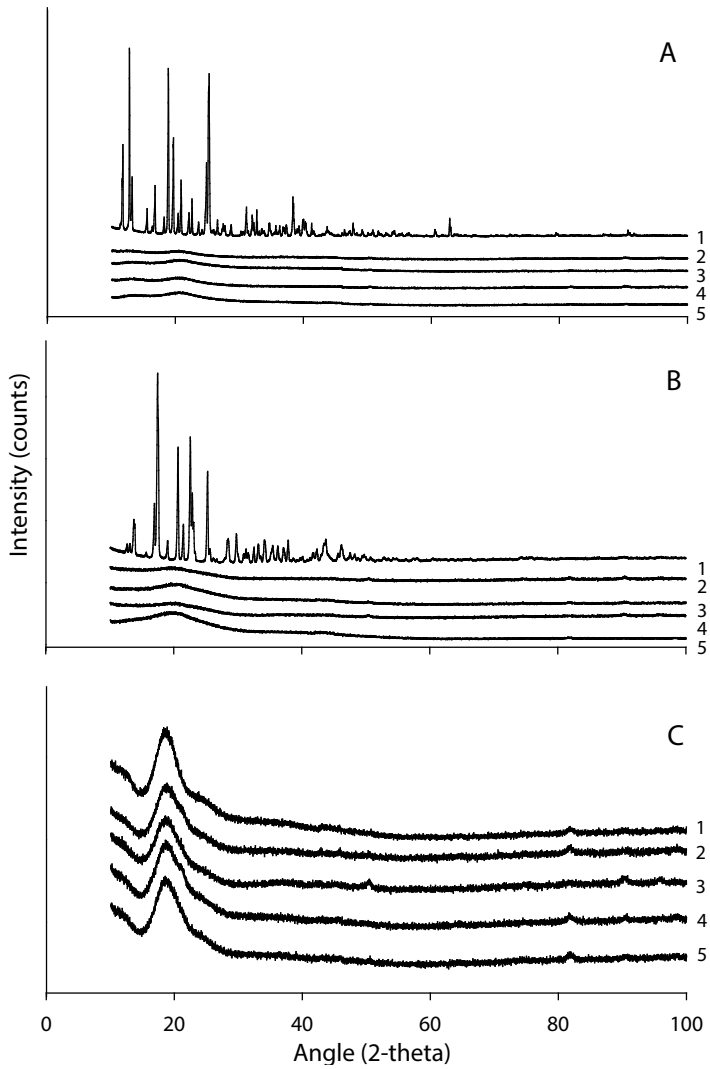

Figure 1: X-ray diffraction patterns of the raw protecting carbohydrates and the dried formulations. A: sucrose formulations, B: trehalose formulations and C: HPβCD formulations. In each panel, 1 represents the raw carbohydrate, 2 and 3 respectively are the freeze-dried and spray-dried formulations without internal carbohydrate, 4 and 5 respectively are the freeze-dried and spray-dried formulations with 10% internal carbohydrate.

The T_g of the sucrose formulations is approximately 36 ºC, the freeze-dried formulations having a slightly higher T_g compared to their equivalent spray-dried formulations (Table 2). This is in correspondence with the slightly higher water content of the spray-dried formulations. The T_g of all dry trehalose formulations is 50 ºC (Table 2). Apparently, the slight increase in water content does not affect the T_g of these formulations, which is a unique property of trehalose that has been reported before (49-51). However, both disaccharide powders appeared to be very hygroscopic and instantly turned into its rubbery state upon

exposure to air, due to increased moisture levels and thereby reduction of the T_g to values below room temperature (52,53). This was not seen in the HPβCD formulations, since the T_g of the HPβCD formulations was found to be over 100 ºC (Table 2).

Size and size distribution

Liposomal solutions containing 100 nm PLP-PEGylated liposomes are opalescent, and exhibit a characteristic red glow when inspected against visible light. This was seen in all formulation solutions before drying, and was confirmed by DLS measurement (Table 1). Reconstitution of the disaccharide-containing PLP-PEGylated liposomes took 40 to 75 minutes with manual shaking, while the reconstitution of the freeze-dried formulations and the spray-dried formulation containing HPβCD was complete within 5 minutes of manual shaking. Upon rehydration of all spray-dried formulations the opalescence and red glow re-appeared, an indication for the presence of 100 nm liposomes. However, with the exception of the HPβCD formulations, the formulations remained turbid upon reconstitution, indicating that also larger particles are present. Indeed this observation was confirmed by DLS analysis, which showed that the mean liposomal size as well as the PDI of all spray-dried formulations were significantly increased, with exception of the HPβCD formulations (Table 3). The PLP-PEGylated liposomal formulations containing HPβCD as lyoprotectant showed only a minor increase in size and PDI after reconstitution. This finding was confirmed by TEM analysis which showed the presence of uniform sized liposomes in the HPβCD formulations after spray- and freeze-drying, irrespective of the presence or absence of internal HPβCD (Figure 2).

Table 3: Effect of the drying method on the size (z-average) and size distribution of the liposomes. Liposomal formulations were prepared with and without the protecting carbohydrate present in the liposomal core (10% internal sugar and no internal sugar)

Formulation		Mean size ø (PDI)		
		Before drying	**After spray-drying**	**After freeze-drying**
No internal sugar	Sucrose	100 nm (0,05)	664 nm (0,76)	94 nm (0,11)
	Trehalose	100 nm (0,03)	1102 nm (0,64)	100 nm (0,15)
	HPβCD	112 nm (0,04)	138 nm (0,18)	107 nm (0,12)
10% internal sugar	Sucrose	104 nm (0,09)	503 nm (0,88)	95 nm (0,07)
	Trehalose	98 nm (0,10)	1159 nm (0,66)	101 nm (0,11)
	HPβCD	104 nm (0,01)	132 nm (0,14)	108 nm (0,17)

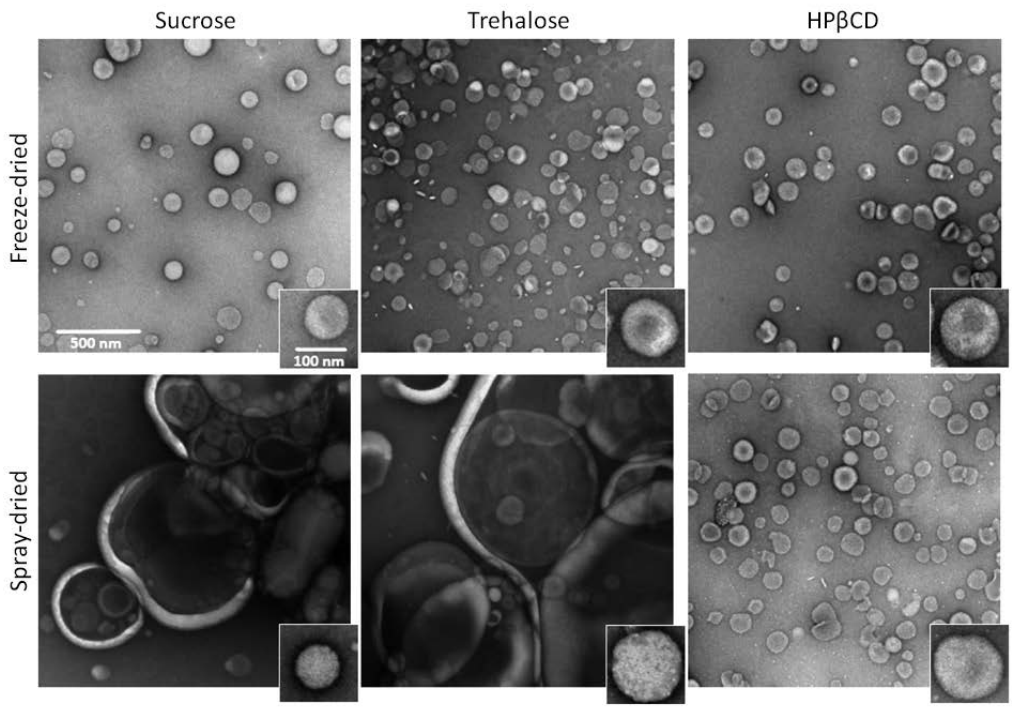

Figure 2: TEM measurement confirms the presence of 100 nm liposomes in all formulations. No aggregates are formed in the freeze-dried formulations containing sucrose, trehalose or HPβCD, as well as in the spray-dried formulation containing HPβCD. Aggregation and rupture of liposomes occurred in the spray-dried formulations containing sucrose and trehalose, which explains their turbidity.

PLP leakage

PLP concentrations were measured using HPLC-UV. Before drying >90% of the PLP was encapsulated in the liposomes. Upon reconstitution of the freeze-dried formulations, the total PLP content of the formulation was comparable to the concentration before drying. However, for the sucrose and trehalose containing formulations only about 50% of the drug was still encapsulated in the liposomes, while for the HPβCD containing formulations this was still >90% (see Figure 3).

For the spray-dried formulations, the results for drug retention were comparable to freeze-drying. For the HPβCD containing formulations approximately 100% of this drug is still encapsulated in the liposomes, while for the disaccharides up to 70% has leaked out of the liposomes.

The composition of the liposomes that are present in the dried substances was comparable to the liposomes in the aqueous formulation (comparable drug to lipid ratio, molar lipid composition DPPC:DSPE-PEG:CHOL 2.4:0.15:1.0), indicating that the leaking of the drug from

the liposomes was not a result of liposome degradation. Clearly, the disaccharides were not able to stabilize the liposomal membranes during the drying process, resulting in drug leakage.

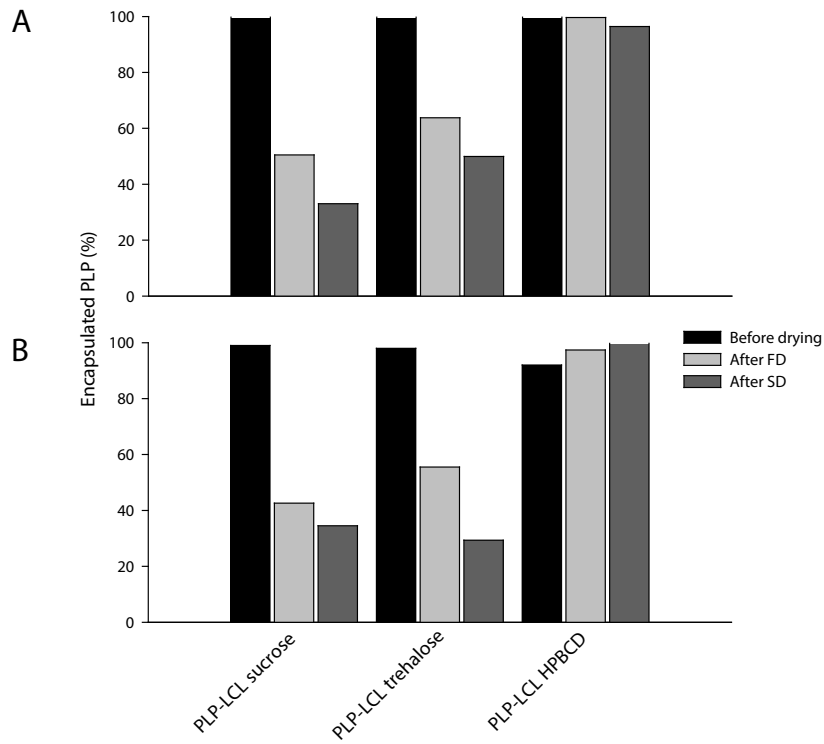

Figure 3: Encapsulated % of PLP in formulations without internal sugar (A) and with 10% internal sugar (B) before drying, after freeze-drying (FD) and after spray-drying (SD)

Discussion

PLP loaded liposomes were prepared in order to compare the stabilizing effect of HPβCD to commonly used disaccharides on the liposomal membranes during drying. The formulations were dried by spray-drying or freeze-drying. Three temperatures are of importance during drying of liposomes: the glass transition temperature (T_g) of the drying formulation, the vitrification temperature (T_g') of the aqueous formulation and the transition temperature (T_m) of the liposomal membrane. The T_g' and the T_g are primarily determined by the lyoprotectant used (14,20,30,48,54). The T_g' is important during freezing of the solution.

Carbohydrates, like disaccharides, are used to protect the liposomal membranes during freezing, by forming a protective amorphous network around the liposomes. This vitrification occurs at temperatures below the T_g' (14). For sucrose and trehalose, the T_g' is -

32 ºC and -30 ºC, respectively (14), while for HPβCD this is about -15 ºC (38). The T_g is important during drying and storage of the formulation. Temperatures higher than the T_g of the (drying) powder could result in collapse of the powder. The T_g of dried sucrose and trehalose (e.g. without residual water) are about 60 ºC and 100 ºC respectively, and decrease with increased moisture content (55,56). The T_g of HPβCD is much higher as compared to the disaccharides (about 220 ºC for dry HPβCD (57)). The T_m mainly depends on the composition of the liposome and is similar for all tested formulations as the same lipid composition was used (Table 1). Due to the high content of cholesterol in the liposomal membrane, the T_m could not be determined (32). However, the T_m of similar liposomes with less cholesterol is typically around 41 ºC in hydrated state (58), and is not expected to change significantly upon dehydration (22,59).

The size and PDI of the liposomes in the spray-dried formulations containing disaccharides increased dramatically, while for the HPβCD formulations no changes in size and PDI were seen. This was also reflected by the 70% of drug leakage from the disaccharide formulations, while for the HPβCD formulations approximately 100% of the drug is still encapsulated in the liposomes. Apparently, spray-drying induces instability of the liposomal membranes that cannot be stabilized by disaccharides. This is likely due to the high drying temperatures obtained during spray-drying. The final T_g of the dried liposomal formulations was 35-50ºC, and has been below these values during the drying process. The outlet temperature of the spray drier was 68ºC, so the drying particles were temporarily heated to temperatures above their T_g. This could have caused transformation of the outer layer of the particles into a viscous liquid state, thereby enhancing its mobility, which could have resulted in partial collapse of the powder particles. The protecting vitrified structure is lost, resulting in instability of the liposomal membranes. Since the T_g of HPβCD is much higher as compared to the disaccharides, collapse of the cyclodextrin formulations is prevented. This indicates the importance of selection of proper settings of the spray-drying parameters. These settings should be based on the temperature characteristics of the selected lyoprotectant and lipid composition used (30).

Previously Hauser and Straus reported no significant structural changes after spray-drying of non-PEGylated small unilamellar vesicles. 90% of the originally entrapped materials remained entrapped in the liposomal cavity during spray-drying, when using sucrose as lyoprotectant (27). Additionally, Chougule et al. developed nanoliposomal dry powder formulations for inhalation, containing tacrolimus and dapsone (28,29). Conventional liposomes of approximately 140 nm were spray-dried with sucrose, trehalose or lactose as lyoprotecting agents at spray-drying conditions comparable to the settings used in our study. Drug retention of 97% for both formulations was reported. However, Wessman et al., showed that size and size distribution increased after spray-drying of 100 nm PEGylated liposomes with lactose as lyoprotectant (17). To the best of our knowledge, drug retention upon reconstitution of spray-dried PEGylated liposomes has not been reported thus far, but

the occurrence of drug leakage in our own study is in line with the results obtained by Wessman et al.

Freeze-drying is performed at lower drying temperatures (up to maximal 0 ºC during secondary drying) and therefore collapse due to exceeding the T_g during drying is not observed. Although no changes in size and PDI were found, approximately 50% of the drug leaked out of the liposomes during freeze-drying of the disaccharide formulations. This indicates that the liposomal membranes have been instable at some timepoint during the drying process or the rehydration with disaccharides as lyoprotectant. In contrast, in the HPβCD formulations no PLP leakage was observed. In freeze-drying, both the formation of ice crystals during freezing and the sublimation of the water from the liposomal surface could cause damage to the liposomal membranes (14,48,60). Due to its higher T_g' the protecting vitrification networks were formed at higher temperatures during freezing of the HPβCD formulations, as compared to the formulations containing the disaccharides. This difference might have resulted in larger ice crystals during freezing of the disaccharide containing formulations, ultimately leading to membrane damage and drug leakage. The drying temperature during primary drying is well below all T_g' values, therefore no differences in membrane stability are expected during primary drying.

Structure bound (adsorbed) water is removed in the secondary drying phase of the lyophilization cycle (14). In this process, the water molecules at the liposomal surface are replaced by molecules of the lyoprotecting agent, to stabilize the membrane structure in the dried state. Carbohydrates replace water by the formation of hydrogen bonds with liposomal surface structures (22,23). Apparently, the lipid:disaccharide ratio of 1:6 (w/w) does not provide sufficient hydrogen replacement to stabilize the PEGylated liposomal membranes, resulting in drug leakage upon reconstitution. HPβCD has a unique structure with a large number of hydrogen donors and acceptors (38,39) and is therefore probably very efficient in stabilizing the liposomal membranes.

Leakage percentages of water-soluble compounds of 0% to up to 60% have been reported when using a variety of disaccharides to stabilize the liposomal membranes during freeze-drying (9,19,23,48,61). According to Crowe and Crowe, 100% drug retention in 100 nm liposomes could be obtained using a lipid:disaccharide ratio (trehalose or sucrose) of approximately 1:4 (w/w). Additionally, they have demonstrated that changes in both the lipid composition and the drying protocol can result in differences in the stability of the dried liposomes (23). To the best of our knowledge, no drug leakage results from freeze-dried PEGylated liposomes have been reported thus far.

HPβCD can form complexes by inclusion of lipophilic drugs into its cavity. The formation of a drug-HPβCD complex potentially can affect the pharmacokinetic profile of the drug, and therefore the drug release profile of the liposomal formulation should be investigated when HPβCD is added to the formulation. The effect of this possible complex formation on the

pharmacokinetic behavior of the formulation is very much depending on the strength of the complexation. Although HPβCD is not able to form a complex with the liposome itself (diameter cavity HPβCD is 6-6.5Å, whereas diameter liposome is 100 nm), complexation of drug entrapped in the liposomal core is possible when using HPβCD as internal lyoprotectant. Indeed, complexation of prednisolone (the underivatized glucocorticoid) with HPβCD has been described (62). However, based on a complexation constant of 4300 $L.mol^{-1}$ for prednisolone, it is very unlikely that the much better water-soluble PLP forms a more stable complex with HPβCD in solution and be of any significance with respect to its pharmacokinetic profile. Nonetheless, it could be advised to stabilize the liposomal membranes only externally with HPβCD to prevent complex formation, since no differences were observed in stability of the formulations with and without internal lyoprotectant.

With respect to the storage conditions of the dried liposomal formulations, a storage temperature of 20-50ºC below the T_g is required. Therefore, the disaccharide containing formulations have to be stored at least refrigerated (+2-8ºC) (63-65). Additionally, the hygroscopic behavior of the dried disaccharide formulations requires them to be stored under cold and dry conditions. (52,53). Since the T_g of the HPβCD formulations is over 100ºC, these formulations can be stored at room temperature.

Conclusion

In conclusion, HPβCD has proven to stabilize the liposomal membranes during both spray drying and freeze-drying. Likely, its relatively high T_g' protects the membranes against damage by ice crystal formation during the freezing phase of the lyophilization cycle, while its relatively high T_g prevents the drying powder from collapse during spray-drying. Additionally, the large number of hydrogen donors and acceptors in the structure of HPβCD likely attributes to the efficiency of replacement of the water molecules at the liposomal surface during drying of the formulation, thereby protecting the liposomal membranes from damage and keeping its structure intact.

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Chapter 3

Preclinical and clinical studies with liposomal glucocorticoids

Chapter 3.1

Safety of glucocorticoids can be improved by lower yet still effective dosages of liposomal steroid formulations in murine antigen-induced arthritis: comparison of prednisolone with budesonide

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Abstract

The goal of this study was to compare the effects of liposomal and free glucocorticoid formulations on joint inflammation and activity of the hypothalamic-pituitary-adrenal (HPA) axis during experimental antigen-induced arthritis (AIA). A dose of 10 mg/kg liposomal prednisolone phosphate (PLP) gave a suppression of the HPA-axis, as measured by plasma corticosterone levels in mice with AIA and in naïve mice. In a subsequent dose-response study, we found that a single dose of 1 mg/kg liposomal prednisolone phosphate (PLP) was still equally effective in suppressing joint inflammation as 4 repeated once-daily injections of 10 mg/kg free PLP. Moreover, this dose gave 22% less suppression of corticosterone levels than 10 mg/kg of liposomal PLP at day 14 of the AIA. In order to further optimize liposomal glucocorticoids, we compared liposomal PLP with liposomal budesonide phosphate (BUP) (1 mg/kg). At 1 day after treatment, liposomal BUP gave a significantly stronger suppression of joint swelling than liposomal PLP (lip. BUP 98% versus lip. PLP 79%). Both formulations also gave a strong and lasting suppression of synovial infiltration in equal amounts. However, at day 21 after AIA, liposomal PLP still significantly suppressed corticosterone levels, whereas this suppression was not longer significant for liposomal BUP. Conclusion: Liposomal delivery improves the safety of glucocorticoids by allowing for lower effective dosing. The safety of liposomal glucocorticoid may be further improved by encapsulating BUP rather than PLP.

Introduction

Glucocorticoid is a powerful anti-inflammatory drug that is widely applied to treat rheumatoid arthritis (RA). Recurrent and high dosing of glucocorticoids however can lead to a plethora of adverse side effects including bone demineralisation, metabolic syndrome and suppression of the hypothalamic-pituitary-adrenal (HPA) axis (1). Therefore, modifications in glucocorticoid compounds, treatment strategy and/or delivery are aimed at improving potency and availability of glucocorticoids, while decreasing their side effects.

Delivery of glucocorticoids within long-circulating 'stealth' liposomes offers a way in which the circulation time of glucocorticoids can be improved (2). Liposomes sized to a diameter up to 100 nm passively extravasate in areas of increased vasodilation e.g. due to local inflammation. In earlier studies, we and others found that a single dose of liposomal glucocorticoid is more effective than repeated doses of free glucocorticoid in several models of inflammation (3-5). The targeting effect may thus allow for less frequent dosing, which was demonstrated in a murine collagen type II arthritis, where a dose of 10 mg/kg liposomal PLP was more effective than 5 repeated once-daily injections of 10 mg/kg free PLP (6). Furthermore, a clinical trial on long-circulating liposomal PLP in RA patients recently demonstrated their safety and efficacy (7).

Although the pharmacokinetics and biodistribution of liposomal glucococorticoid delivery may explain their strong anti-inflammatory effect, less is known about the side effects of liposomal glucocorticoids. In animal models of experimental arthritis, acute side effects like weight loss and a suppression of the HPA-axis can be studied as indication for the side effects of glucocorticoids (8).

The production of endogenous glucocorticoids (corticosterone in mice) by the adrenal cortex is stimulated by adrenocorticotropic hormone (ACTH) produced and released by the pituitary gland, which in turn is regulated by corticotrophin releasing hormone (CRH) produced by the hypothalamus. The activity of the HPA-axis is regulated via a feedback mechanism of its end product, glucocorticoids, to the hypothalamus. Thus, activity of the HPA-axis is reflected by the levels of circulating glucocorticoids in the blood (9).

Encapsulating different glucocorticoids into liposomes has been suggested as a way to optimize their efficacy. In most studies on liposomal glucocorticoid delivery, PLP was used, however, encapsulation of other glucocorticoids may be more effective. Of several tested liposomal glucocorticoids in a murine melanoma model, budesonide phosphate (BUP) was the most efficacious formulation in inhibition of tumor growth in these mice (10). Budesonide is a potent glucocorticoid that is more effective at lower doses than prednisolone in the treatment of human RA (11). In addition, budesonide is cleared relatively rapidly from systemic circulation compared to prednisolone in their free form in humans (12). Currently, budesonide is mostly used as treatment for asthma (13), however, its

characteristics make it an ideal candidate for liposomal delivery as treatment for RA as it may further reduce the required dose of liposomal glucocorticoid whereas its systemic availability is kept to a minimum due to its rapid systemic clearance.

The goal of this study was to compare the efficacy of liposomal PLP and BUP on joint inflammation during antigen-induced arthritis and investigate their effects on the HPA-axis.

Materials and methods

Preparation of liposomal glucocorticoids

Liposomes were prepared as described previously (10). Briefly, a lipid formulation of dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshave, Germany), PEG 2000-distearoyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0 were dissolved in ethanol which was then evaporated from a round-bottom flask to create a lipid film. The lipid film was hydrated in water to create empty liposomes or in a solution of 100 mg/ml prednisolone disodium phosphate (PLP) (Bufa, Uitgeest, the Netherlands) in water to create liposomal PLP or the lipid film was hydrated in Budesonide Phosphate in water to create liposomal BUP. Single Unilamellar Vesicles were obtained by filtering the liposomal dispersion multiple times through polycarbonate filter membranes decreasing in pore diameter until the liposomes had a mean diameter in the range of 90-110 nm with a polydispersity of <0.2. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern ltd., Malvern, UK). Unencapsulated PLP was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Encapsulation dose of PLP was determined by extracting the aqueous phase from liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content. With ultra performance liquid chromatography (UPLC) (14,15), using a RP18 (5 µm) column (Merck) and a mobile phase acetonitril-water with pH of 2, connected to an UV-detector, which was set at 254 nm, both prednisolone or budesonide and its phosphate ester could be measured in one single run. The detection limit for the UPLC setup was 20 ng/ml. Liposomal preparations contained 1–10 mg/ml glucocorticoid (varying slightly between batches) and an average of 60 µmol phospholipid.

Animals

Mice (male C57Bl/6) were purchased from Elevage-Janvier (Le Genest Saint Isle, France) and were housed in filter-top cages and fed a standard diet and water ad libitum. All animal procedures were approved by the institutional ethics committee.

Antigen-Induced Arthritis

The antigen-induced arthritis (AIA) was performed as decribed previously (16). Briefly, mice at an age of 8-12 weeks were immunized with 100 µg methylated bovine serum albumin (mBSA, Sigma-Aldrich, St Louis, USA), emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, USA) which was injected into the flanks and the footpath of the forelegs. Heat-killed *Bordetella pertussis* (RIVM, Bilthoven, the Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with in total 50 µg mBSA/Freund's complete adjuvant were given in the neck region 1 week after the initial immunization. Two weeks after these injections, AIA was induced by intra-articular injection of 60 µg of mBSA in 6 µl of phosphate-buffered saline into the knee joints. Mice were weighed at the beginning and at the end of the AIA. Body weight is expressed as percentage of their body weight at the start of the AIA.

Treatment, sacrifice and tissue isolation

At day 3 when severe arthritis had developed mice were treated with either either a single injection of liposomal glucocorticoid, repeated daily injections of 10 mg/kg free PLP or PBS. At day 14 and 21 after AIA-induction, whole blood was obtained by retro-orbital bleeding of mice anaesthetized with isoflurane (5%), into MiniCollect tubes (Greiner bio-one). Hereafter, mice were sacrificed by cervical dislocation and whole knee joints were stored in formalin for histology. After centrifugation of whole blood samples, plasma samples were stored at -80° C.

Measurement of corticosterone

Corticosterone (B) was measured by radio-immuno assay from blood plasma samples as described by Sweep *et al*. (17). Briefly, plasma B was measured by RIA after extraction using antiserum raised in sheep against a B-21-hemisuccinate-BSA conjugate. To each plasma sample (50 µl), 100 µl 0.1 N NaOH, 100 µl B tracer ([1α,2α-N-^{3}H]B; Amersham International PLC, Amersham, Aylesbury, Buckinghamshire, United Kingdom; 10,000 dpm in 0,2% ethylene glycol (Merck)/water) and 500 µl bidistilled water were added. Extraction was carried out using 7.5 ml dichloromethane (Baker, Deventer, The Netherlands). The water phase was discarded and the dichloromethane phase was evaporated. The residue in 2 ml 0.2% EGW and mixed with 200 µl of an antiserum dilution (final dilution 1:100,000) and 100 µl B tracer (10,000 dpm/tube). Antiserum and tracer were diluted in 0.05 M borate buffer (pH 8.0) containing0.1% human γ-globulin (Beriglobin S, Behring, Marburg, Germany). Aliquots of 300 µl eluate were taken for recovery. Amounts of 0-5000 fmol/tube B (steraloids, Inc., Wilton, NH) diluted in 0.2% EGW were used for obtaining a standard curve. After incubation overnight at 4° C, the suspension was mixed with 150 µl dextran-coated charcoal suspension and left at 4° C for 5 min. After centrifugation, the supernatants were decanted into counting vials, and 4 ml Aqualuma (Lumac LSC, Olen, Belgium) was used as counting

solution for radioactivity measurements. The sensitivity of the assay was 25-45 fmol/tube. The within- and between-assay coefficients of variation were 10.0% and 16.7%, respectively.

Measurement of enzymes in blood plasma

The enzymes lactate dehydrogenase (LD), alkaline phosphatase (AP), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were measured by turnover of their respective substrates by spectrophotometry on a Modular spectrophotometer (Roche Diagnostics) (18).

Histology

Total knee joints of mice were isolated after sacrifice and fixed for 4 days in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Standard frontal sections of 7 µm were mounted on superfrost slides (Menzel-Gläser, Braunschweig, Germany) for histology and immunostaining. Histology was performed on sections stained with hematoxylin and eosin (HE). The severity of joint inflammation was determined as described previously (19), by scoring the amount of cellular infiltration into the synovium using an arbitrary scale (0–3), for three representative knee joint sections for each mouse (5 mice for each treatment group). Scoring was performed in a blinded manner by two independent observers: 0, no cells; 1, mild cellularity; 2, moderate cellularity; 3, maximal cellularity.

Statistical analysis

Statistical significance of the differences between glucocorticoid-treated groups and control-treated (PBS) groups were tested by Mann-Whitney U-test (non-parametric test) with the aid of Graphpad Prism 5.0 software. Data are expressed as mean ± standard error of the mean (SEM), P-values less than 0.05 were considered as statistically significant.

Results

Liposomal PLP reduces activity of the HPA axis.

To study the side effects of liposomal and free PLP, we measured blood plasma levels of corticosterone, indicative for activity of the hypothalamic-pituitary-adrenal (HPA) axis. In previous studies we found that a single dose of 10 mg/kg liposomal prednisolone phosphate (PLP) strongly suppressed joint inflammation in experimental arthritis and was much more effective than 4 repeated once-daily injections of free PLP (6).

During AIA, plasma levels of corticosteroids strongly increase and were 240% higher at day 14 after arthritis induction when compared to naïve mice. Treatment of AIA with single dose liposomal PLP and multiple dose (4x10 mg/kg) free PLP suppressed mean plasma

corticosterone levels by 49% (from 863 to 456 pmol/ml) and 28% (to 634 pmol/ml) respectively (Figure 1).

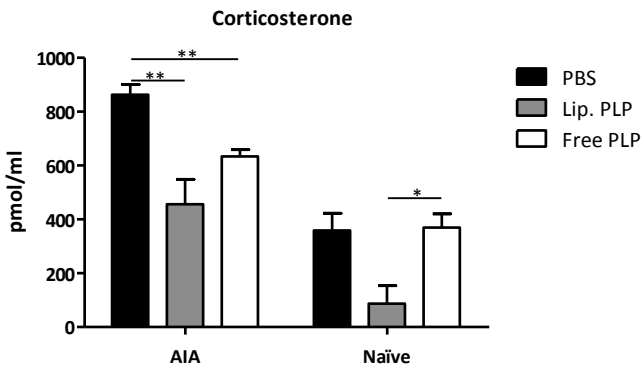

Figure 1: Liposomal PLP suppresses corticosterone levels during AIA and in naïve mice. Mice were injected with liposomal PLP (10 mg/kg), free PLP (4x10 mg/kg) or PBS (control). Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05; **P<0.01.

Because activity of the HPA-axis is strongly related to disease activity, we also studied the side effects of glucocorticoids in naïve mice in which the arthritis is not additionally influenced by effects of experimental arthritis. Here we found a reduction in mean plasma levels of corticosterone of 76% by single dose liposomal PLP (from 358 to 87 pmol/ml) when compared to mice injected with PBS. In contrast, multiple dose free PLP did not suppress corticosterone in naïve mice, when compared to PBS injection, indicating that the 10 mg/kg dose of liposomal PLP, which is very effective in several arthritis models, causes suppression of the HPA-axis.

Liposomes are largely taken up from the blood by Kuppfer cells in the liver and other cells of the mononuclear phagocytic system. Therefore, we also measured enzymes in blood plasma which are indicative for liver toxicity and cellular stress: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (AP) and lactate dehydrogenase (LD) (Figure 2). In naïve mice, liposomal PLP had minimal effects on ALAT, ASAT, AP and LD (29%, 17% 1% and 6%, respectively, when compared to PBS treatment).

Lower dosing of liposomal PLP maintains efficacy against antigen-induced arthritis

To see whether the single dose liposomal PLP can be lowered, without losing its efficacy compared to multiple dose free PLP, we studied the effect of different doses of liposomal PLP on joint inflammation during antigen-induced arthritis (AIA) in relation to its systemic side effects.

The different single doses of liposomal PLP (10, 5 and 1 mg/kg) all strongly reduced synovial infiltration at day 14 after AIA (11 days after treatment, Figure 3). Suppression of joint inflammation by liposomal PLP occurred in a dose responsive manner, with an average suppression of 75%, 63% and 47% for 10, 5 and 1 mg/kg of liposomal PLP, respectively.

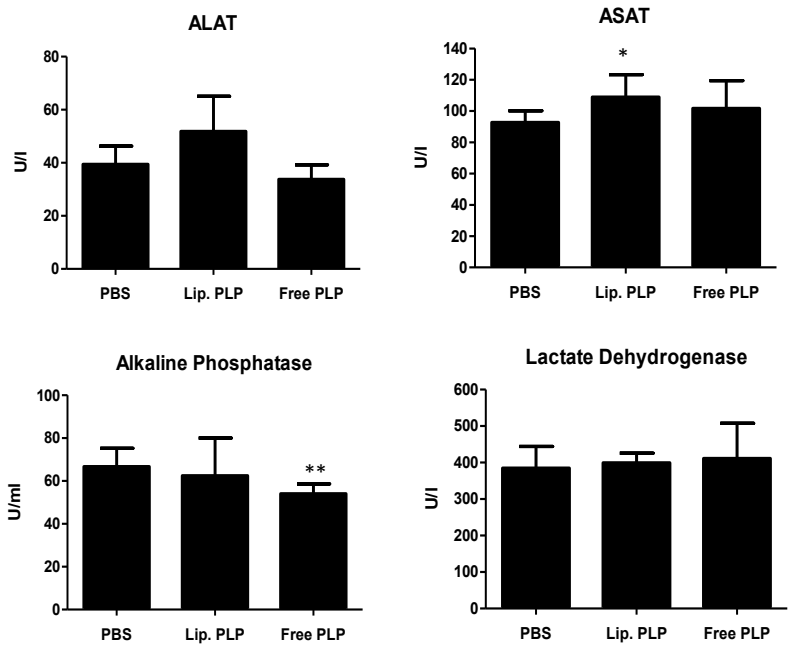

Figure 2: Effect of Liposomal and free PLP on liver toxicity in naïve mice. Healthy mice were injected with Liposomal PLP (10 mg/kg), 4x free PLP (4x10 mg/kg) or PBS (control). Plasma levels of the liver enzymes AP, AD, ALAT and ASAT were determined at day 5 after injection. Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05; **P<0.01.

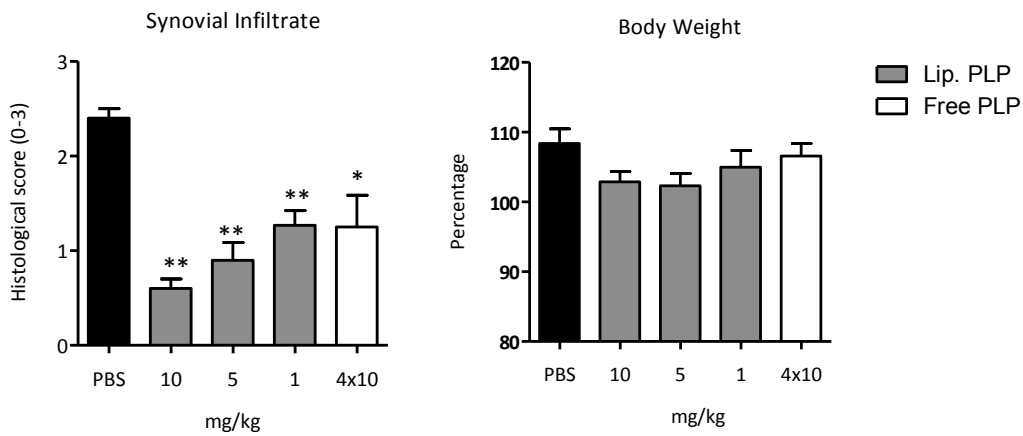

Figure 3: Lower dosing of Liposomal PLP maintains efficacy against AIA. Mice were injected at day 3 of arthritis with 10, 5 and 1 mg/kg of liposomal PLP, free PLP (4x10 mg/kg) or PBS (control). Joint inflammation was determined at day 14 of the AIA. Body weight is expressed as percentage of their weight at time of sacrifice compared to their weight before treatment (day 3). Note that all PLP formulations significantly suppress joint inflammation, without significantly suppressing body weight. Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05; **P<0.01 versus control (PBS) treated group.

Interestingly, a dose of 1 mg/kg liposomal PLP was still comparable in potency as 4 repeated once-daily injections of 10 mg/kg free PLP (48% suppression, Figure 3).

As even lower doses of liposomally delivered PLP still improved their efficacy compared to the free form, we wanted to see how this would affect its systemic side effects and how this would compare against another liposomal glucocorticoid formulation.

Optimization of liposomal glucocorticoid delivery by encapsulating budesonide phosphate

To further optimize the efficacy of liposomal glucocorticoid delivery, we studied the effect of encapsulation of a novel glucocorticoid: budesonide phosphate (BUP). Budesonide is a potent glucocorticoid that has a relatively high clearance rate and may therefore have less systemic side effects than prednisolone. To compare the efficacy of both forms, mice with AIA were treated with 1 mg/kg of liposomal PLP and BUP.

To evaluate the early effects of liposomal PLP and BUP, we measured joint swelling in the knee joint already at day 1 after treatment using ^{99M}Tc-uptake. At this time point, both formulations already significantly suppressed joint swelling when compared to control PBS-treatment (Figure 4). Liposomal BUP proved to be superior to liposomal PLP, with liposomal BUP completely suppressing joint swelling (lip. PLP 79% versus lip. BUP 98%).

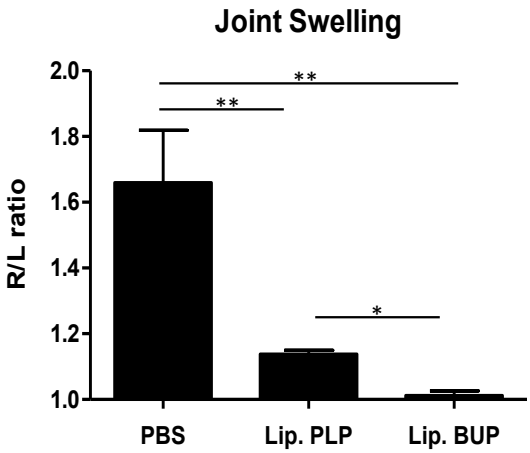

Figure 4: Liposomal PLP and BUP suppress joint swelling during established AIA. Liposomal PLP and Liposomal BUP (1 mg/kg) were injected at day 3 after AIA. Joint swelling was determined by measurement of ^{99M}Tc-uptake at day 1 after treatment. Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05; **P<0.01.

Additionally, we measured a suppression of joint inflammation by histology at day 14 and day 21 after AIA induction (Figure 5). Both formulations were equally potent and showed a strong suppression of joint inflammation at day 14 after AIA (49% and 52% suppression for liposomal PLP and BUP, respectively). Both formulations still suppressed joint inflammation at day 21 after AIA (30% and 29% for lip. PLP and lip. BUP respectively).

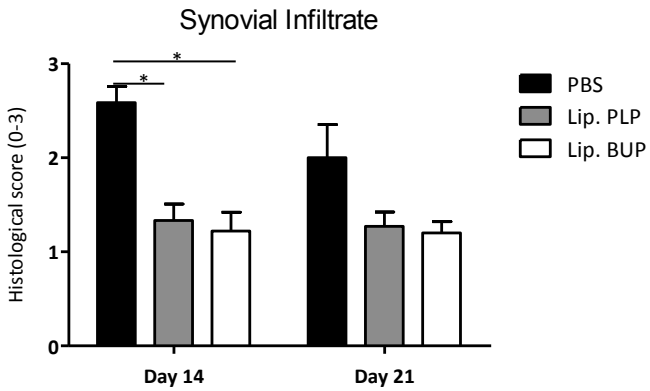

Figure 5: Liposomal PLP and BUP give a lasting suppression of joint inflammation during AIA. Liposomal PLP and Liposomal BUP (1 mg/kg) were injected at day 3 after AIA. Joint inflammation was determined by histological scoring at day 14 and 21 after AIA. Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05.

To evaluate and compare the side effects of liposomal PLP and liposomal BUP at this low dose (1 mg/kg), we again measured corticosterone levels. Firstly, we noted that corticosterone levels in control, PBS-treated mice waned by 39% from day 14 to day 21 after AIA (Figure 6). Secondly, at day 14 of the AIA, liposomal PLP and BUP (1 mg/kg) significantly suppressed corticosterone levels by 24% and 34% from control, PBS-treated mice respectively. At day 21 after AIA, liposomal PLP still showed a low, although significant, reduction in corticosterone levels. In contrast, no significant reduction was detected anymore in the liposomal BUP treated group suggesting a faster recovery of the HPA-axis with this novel formulation.

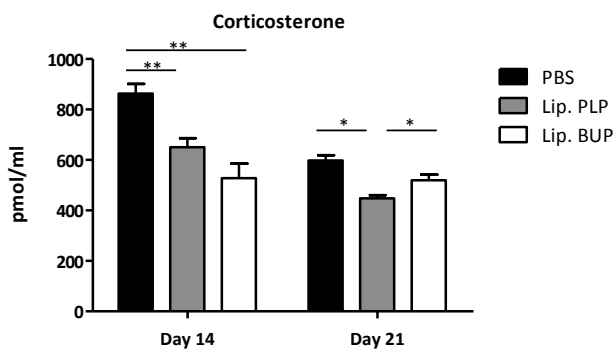

Figure 6: Suppression of the HPA-axis by liposomal PLP and BUP (1 mg/kg). Mice were injected at day 3 after AIA. Note liposomal BUP does not significantly suppress the HPA-axis at day 21 after AIA. Data represent mean ± S.E.M. *(n=5)*. Statistical significance between treatment groups was tested by Mann-Whitney U test. *P<0.05; **P<0.01.

Discussion

In free form, high-, pulse-dosed glucocorticoids cause unwanted side effects in the treatment of rheumatoid arthritis (20). Liposomal delivery offers a way to decrease the side effects and increase the efficacy of glucocorticoids by increasing their circulation time and selective biodistribution towards areas of inflammation, which allows for less frequent drug dosing (6,21). In the present study we found that a single dose of 1 mg/kg of liposomal glucocorticoid was comparable in efficacy with 4 repeated once-daily injections of 10 mg/kg

free PLP. Furthermore, the low dose of liposomal PLP gave a fast and durable suppression of inflammation up to day 21 of the AIA (18 days after treatment).

Liposomes sized to a diameter near 100 nm, reach the inflamed joints via extravasation out of dilated blood vessels, which explains their selective targeting of sites of inflammation. Long-circulating liposomes evade uptake by the mononuclear phagocytic system (MPS) due to the incorporation of poly-ethelene glycol (PEG) in their lipid membrane. However, still a large portion of the liposomes is taken up by MPS cells in spleen and liver (4). In the present study, we found no major effects on enzymes indicative of liver toxicity, despite their pronounced biodistribution to this organ.

Glucocorticoids are a natural product of the HPA-axis and can regulate their own production via feedback signaling to the hypothalamus. When therapeutically administered glucocorticoids enter the systemic circulation, they can regulate (suppress) the activity of the HPA-axis in a manner similar to endogenous glucocorticoids (22).

Studies by Metselaar *et al.* in rats have shown that liposomally encapsulated PLP remains available in the circulation for days, but that PLP is not released from the liposome particles while they are in the circulation (4). Therefore, the plasma concentration of 'free' circulating (i.e. not encapsulated in liposomes) prednisolone is very low. Nevertheless, we observed in the present study a suppression of the HPA-axis by liposomal PLP in naïve mice, which suggests that there are systemic side effects of liposomal glucocorticoids, at least for the highest dose tested. Hypothetically, low plasma levels of 'free' glucocorticoids could be induced after degradation and subsequent release by the MPS cells.

In our study, corticosterone levels in control PBS-treated mice with AIA were 2.4 times higher than in naïve mice, which demonstrates the activation of the HPA-axis in this model. This is in line with earlier studies in which a high correlation between the activity of the HPA-axis and arthritis was found (22). The HPA-axis is stimulated by pro-inflammatory cytokines, like IL-1β, IL-6 and TNF-α, which are produced to a high extend during AIA (17,23-25). The strong suppression of joint inflammation induced by liposomal glucocorticoid treatment is likely to diminish pro-inflammatory cytokine levels and thus explain the reduced activation of the HPA-axis during AIA.

Macrophages contribute for a large part to the activation of the HPA-axis as these cells are the main producers of inflammatory cytokines (24,25). Stealth liposomes, when given systemically, pass the endothelial layer of the blood vessels in the synovium and are directly encapsulated by the macrophages lying around the blood vessels. As these cells are of crucial importance in onset and propagation of antigen-induced arthritis (26), direct targeting by glucocortocids may explain their rapid and strong effects on joint inflammation.

Liposomal glucocorticoid delivery may be further optimized by encapsulating novel glucocorticoids with superior characteristics. Budesonide is a more potent glucocorticoid than prednisolone (27), therefore, liposomal BUP may still be effective at lower dosages than liposomal PLP. As budesonide has been shown to have a relatively short half life in humans and in mice, this may also result in safer use of liposomal glucocorticoid in terms of side effects (28). Furthermore, ACTH response studies in RA patients treated with budesonide and prednisolone showed less suppression of the HPA axis with budesonide, whereas it had increased potency over prednisolone (29). Administration of slow-released oral budesonide via the ileum was previously unsuccessful in RA patients (30), however, liposomal encapsulation specifically reaches inflamed joints and may therefore be more successful. Moreover, the results in the present study demonstrate that liposomally delivered BUP is more potent than liposomal PLP in reducing joint swelling and in addition allows for a faster recovery of the HPA-axis. A high potency of liposomal BUP combined with a rapid systemic clearance thus make liposomal budesonide phosphate a good candidate for future therapy against RA.

Conclusions

Liposomal delivery improves the safety of glucocorticoids by allowing for lower effective dosing. The safety of liposomal glucocorticoid may be further improved by encapsulating BUP rather than PLP.

Acknowledgements

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Chapter 3.2

Long-circulating liposomal prednisolone versus pulse intramuscular methylprednisolone in patients with active rheumatoid arthritis

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Abstract

Objective: Glucocorticoids (GCs) are potent anti-inflammatory drugs but their use in rheumatoid arthritis (RA) is limited by poor target localization and systemic exposure leading to toxicity. Targeted delivery to the site of inflammation with GCs encapsulated in long-circulating liposomes (LCL) can improve the therapeutic index. This approach has proven successful in animal models of arthritis, but has not yet been studied in humans. The present study was aimed to assess the safety and therapeutic effect of a single, intravenous administration of long-circulating liposomal prednisolone (LCLP) in patients with active RA.

Methods: In 6 patients an open label dose-escalating study was performed to assess toxicity of LCLP. A subsequent cohort of 16 patients was randomized to a single dose of 150 mg LCLP or a single intramuscular administration of 120 mg methylprednisolone control medication. The safety profile of LCLP was determined by the occurrence of adverse events (AE) during treatment and follow-up. The therapeutic efficacy was measured using weekly DAS 28 and VAS score assessments.

Results: The safety analysis showed comparable pattern of AEs in both treatment groups. There was one serious adverse event (infusion reaction) probably related to the study medication. DAS 28 scores improved better in the LCLP group. VAS scores showed a faster improvement in the LCLP group compared to the reference group. Only LCLP-treated patients showed responses that fall in the EULAR category 'good response'.

Conclusion: LCLP is safe and efficacious in the therapy of active RA. Larger studies aimed to explore its efficacy are warranted.

Introduction

Rheumatoid arthritis (RA), is a chronic, progressive, and debilitating disease often leading to disability (1,2). Prednisolone and other glucocorticoids (GC) can be highly effective in treating joint inflammation, but their systemic application is limited because of a high incidence of adverse effects (AE) including osteoporosis, hypothalamic-pituitary-adrenal axis (HPA) suppression, muscle wasting, insulin resistance, easy skin bruising, increased risk of serious bacterial infections (3,4), and cardiovascular events (5). In most cases the severity of these AE depends on dose, duration of exposure and potency of the prescribed agent. Besides a poor safety profile, also poor localization in inflamed areas in the body limits the usefulness of GC in the patient, as this requires frequent administration of GC to attain adequate therapeutic benefit (6).

In recent years, several lines of investigation have been pursued to improve the therapeutic index of GC (7). These lines encompass for instance the development of selective GC receptor agonists (SEGRAs) (8-11), the combination of GC with drugs that potentiate their effects in activated inflammatory cells (12,13), the development of controlled-release formulations (14), and the design of advanced formulations that achieve targeted delivery of GCs to the actual sites of inflammation (15-18).

Targeted delivery of GCs can be realized by encapsulation in long circulating liposomes (LCL) that circulate after i.v. injection and at the same time extravasate at the sites of inflammation lesions by virtue of the increased vascular permeability, building local depots of GC selectively at the target sites. This approach proved to be highly effective in preclinical studies with experimental animal models of arthritis (17,18), other inflammatory diseases and even cancer (15,16). Clinical studies with identical, radiolabeled LCL, but without encapsulated drug, have shown that the approach of selective GC delivery to arthritic joints by LCL may also apply to humans (19-21). Herewith we present the first clinical study with targeted delivery of GC by LCL in patients with active RA.

Patients and methods

Patients

A total of 22 consenting patients with RA were enrolled in the present study. Criteria for eligibility were as follows: age ≥ 18 years, RA according to the revised 1987 ARA criteria (22), active disease as defined by a Modified Disease Activity Score (23) (DAS 28) ≥ 3.2 at the screening visit and the need for bridging therapy with systemic GCs according to the rheumatologist in care of the patient.

Exclusion criteria included abnormal renal, liver or hematological tests, current pregnancy, breastfeeding, infections or malignancies, clinically severe or unstable medical conditions

and endocrine disorders. Oral GCs were not permitted within 2 weeks prior to study entry, intra-articular or intramuscular GCs were not allowed within 8 weeks prior to baseline and therapy with disease modifying anti-rheumatic drugs (DMARD) had to be stable within 12 weeks prior to trial initiation.

The study was performed at the Radboud University Nijmegen Medical Center, The Netherlands and approved by the Local Ethics Committee Arnhem-Nijmegen, The Netherlands.

Treatment protocol

This pilot, dose-escalating study started with an open-label cohort aimed to assess potential toxicities of low dose LCLP. The first six patients received a single dose of 37.5 mg (n = 3) and 75 mg (n = 3) prednisolone disodium phosphate-containing LCL intravenously (equivalent to 30 and 60 mg methylprednisolone acetate respectively). Thereafter, in the double-blinded phase, patients were randomized to receive either a single dose of 150 mg prednisolone disodium phosphate-containing LCL intravenously and placebo intramuscularly or a single dose of 120 mg methylprednisolone acetate intramuscularly and placebo intravenously. Dose escalation was allowed if the former cohort was completed without significant AE.

The dose of methylprednisolone chosen in our study is frequently used as bridging therapy to treat short-term flares of RA. Prednisolone disodium phosphate was chosen since it encompasses a water-soluble phosphate ester group, which is required for stable encapsulation in the aqueous phase of the LCL. The chosen dose of prednisolone is based on the fact that the relative anti-inflammatory effects of methylprednisolone and prednisolone are 5 and 4 fold respectively as compared to hydrocortisone. After satisfying the in- and exclusion criteria, the administration of the study medication was planned. On day 1, patients were admitted to the ward where they received prednisolone containing LCL and placebo or methylprednisolone and placebo. Since complement-mediated adverse events have been reported during i.v. administration of LCL (24,25), the infusion rate for the LCLP/placebo was low and slowly accelerated. In case of infusion related toxicities, the infusion was withheld and, if needed, 2 mg clemastin was administered.

Follow-up

After baseline, patients were assessed weekly for up to 12 weeks. Each visit included clinical evaluation, assessment of the disease activity, vital signs, safety assessments, and blood sampling. The disease activity was measured by the same assessor using the Disease Activity Score (DAS28) and Visual Analogue Scale (VAS) (23), and the response to therapy, using the European League Against Rheumatism (EULAR) criteria (26, 27). Disease flare was defined by an increase of the DAS28 of > 1.2 or an increase of the DAS of 0.6-1.2 if this resulted in a

DAS28 of > 5.1, on the weekly assessments (28). The decision to perform a new therapeutic intervention during the 12 week follow-up was based on clinical grounds (RA disease activity) and on the decision of the patients and the rheumatologist.

Laboratory evaluations included measurement of the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), hemoglobin, platelet and white blood cell (WBC) counts, serum creatinine and liver function tests. Fasting glucose, insulin levels and serum lipoproteins were measured every two weeks. Serum osteocalcin and urine N-telopeptide were assessed at baseline and at week 6 and 12. Serum concentrations of prednisolone disodium phosphate, prednisolone, methylprednisolone and cortisol were measured immediately after administration of the study medication and at day 2, 4 and 7.

Trial medication

LCLP consists of PEGylated liposomes, i.e. small (< 150 nm) phospholipid vesicles coated with polyethylene glycol (PEG) (so-called long-circulating liposomes (LCL) because of their longevity in the blood circulation after i.v. injection). The lipid bilayer encloses an aqueous compartment in which the water-soluble disodium phosphate derivative of prednisolone is entrapped. Each mL formulation contains 1.5 mg prednisolone sodium phosphate (BUFA, Uitgeest, The Netherlands), 30 mg palmitoyl phosphatidyl choline (DPPC), 9 mg distearoyl phosphatidyl ethanolamine-PEG2000 (PEG-DSPE) (both from Lipoid GmbH, Ludwigshaven, Germany), and 8 mg cholesterol (BUFA, Uitgeest, The Netherlands). The liposomes are dispersed in 10% sucrose buffered with phosphate buffer at a pH of 7.4.

LCLP is prepared by mixing the lipid constituents with an aqueous solution of the GC followed by repeated high-shear homogenization to reduce the size of the formed vesicles. Unencapsulated GC is removed by tangential flow filtration. Sterilization takes place by dead-end filtration using 0.2 micrometer filter membranes (Sartorius, Nieuwegein, The Netherlands).

LCLP is subject to the following characterization and quality controls: particle size and polydispersity index (around 100 nm and <0.1 respectively as measured by dynamic light scattering), content of prednisolone and lipid excipients as measured by HPLC assays, sterility and pyrogenicity (the latter determined with the LAL assay (Biowhittaker, Walkersville, MD)), and solvent residual testing. All raw material purchased was GMP-certified (GMP, Good Manufacturing Practice) and the liposome manufacturing was performed under GMP conditions.

LCLP and methylprednisolone acetate (Depo-Medrol, Pfizer BV, Capelle a/d Ijssel, The Netherlands) control medication were blinded and prepared at the Pharmacy Department of the Radboud University Nijmegen Medical Center.

Data analysis

Clinical data are expressed as the mean ± SD unless stated otherwise. The DAS 28 score was the primary outcome measure to test the efficacy of the trial intervention. Type I error was controlled at a significance level of 0.05 for the analysis of the primary outcome. Several secondary efficacy measures were analyzed to confirm the findings of the primary measure. These included the individual components of the DAS, the patient assessment for pain, the physician assessment for disease activity. We did not perform a multiplicity adjustment since the intention of the study is not to assess the secondary measures at the same significance level as the primary outcome. As this is a trial with a limited number of patients, most analyses were descriptive only. If a patient experienced a disease flare or showed no response to medication according to the EULAR criteria, a second intervention could take place. Where statistic analysis could be applied the two sample t-test was used.

Bioanalysis

Blood samples for determination of prednisolone phosphate, prednisolone, and methylprednisolone plasma concentrations were taken before, immediately after, and at day 1, 2, 4, and 7 after treatment. The samples were centrifuged to obtain plasma and stored at –80 ^{o}C. The compounds to be measured were extracted by organic extraction after addition of ammonium carbonate and assayed with a HPLC-method involving a gradient program of aqueous acetonitrile mixtures. The detection limit of the method was 20 ng/ml.

Results

Baseline demographic and clinical characteristics

A total of 22 patients were enrolled between 30 August 2005 and 8 January 2008. All subjects were followed up according to the protocol and completed the study. The patient demographics are shown in Table 1.

At baseline there were differences between the markers for the severity of RA. In the test medication 5/8 patients were Rheumatoid factor positive compared to 7/8 in the reference group. The ESR C-reactive protein levels and DAS were substantially lower in the LCLP group.

Two patients were excluded from the efficacy analysis: one subject in the control group had been treated with i.m. methylprednisolone within 8 weeks prior to the baseline visit, and with one patient in the treatment medication group the infusion of trial medication was stopped after 15 minutes due to a (pseudo)allergic reaction.

Table 1: Demographics and baseline characteristics

	Statistic	Stage 1: 37.5 mg N = 3	Stage 1: 75 mg N = 3	Stage 2: Test Medication N = 8	Stage 2: Reference Medication N = 8
Age (years)	Mean (SD)	46.7 (2.52)	50.0 (14.93)	57.4 (12.15)	56.1 (10.37)
	Median (range)	47.0 (44 49)	44.0 (39 67)	62.0 (41 70)	54.5 (40 72)
Gender					
Male	% (n/N)	66.7% (2/3)	66.7% (2/3)	50.0% (4/8)	50.0% (4/8)
Female	% (n/N)	33.3% (1/3)	33.3% (1/3)	50.0% (4/8)	50.0% (4/8)
Rheumatoid Factor					
Positive	% (n/N)	66.7% (2/3)	100% (3/3)	62.5% (5/8)	87.5% (7/8)
Disease Activity at baseline:					
ESR	Mean (SD)	8.7 (9.9)	42.3 (13.6)	17.4 (12.3)	36.6 (37.7)
CRP	Mean (SD)	5.0 (0.0)	34.0 (9.5)	17.9 (15.4)	42.5 (44.7)
DAS	Mean (SD)	5.1 (1.6)	6.8 (0.9)	5.0 (1.5)	6.2 (1.0)

Clinical response and treatment

Figure 1 shows the DAS28 score. Patients receiving a second intervention during the follow up were not longer evaluated for disease activity. A substantial difference between the two treatment groups in terms of mean DAS28 score at baseline was observed (5.0 for the test medication group and 6.2 for the reference medication group). For this reason the DAS28 results are also expressed as percentage improvement relative to the baseline value (Figure 1B). A pronounced therapeutic improvement is visible between baseline and the first visit in the test medication group during the first weeks after treatment. In the reference medication group a slower therapeutic improvement is visible. The difference is significant at week 1 after treatment.

EULAR Response

Figure 2 shows the distribution of patients achieving a good, moderate or no EULAR response after intervention. Patients receiving a second intervention during the follow up were no longer evaluated for disease activity. The test medication group shows a higher percentage of responders in weeks 1 and 2. Interestingly, only patients in the test medication group experienced a good EULAR response.

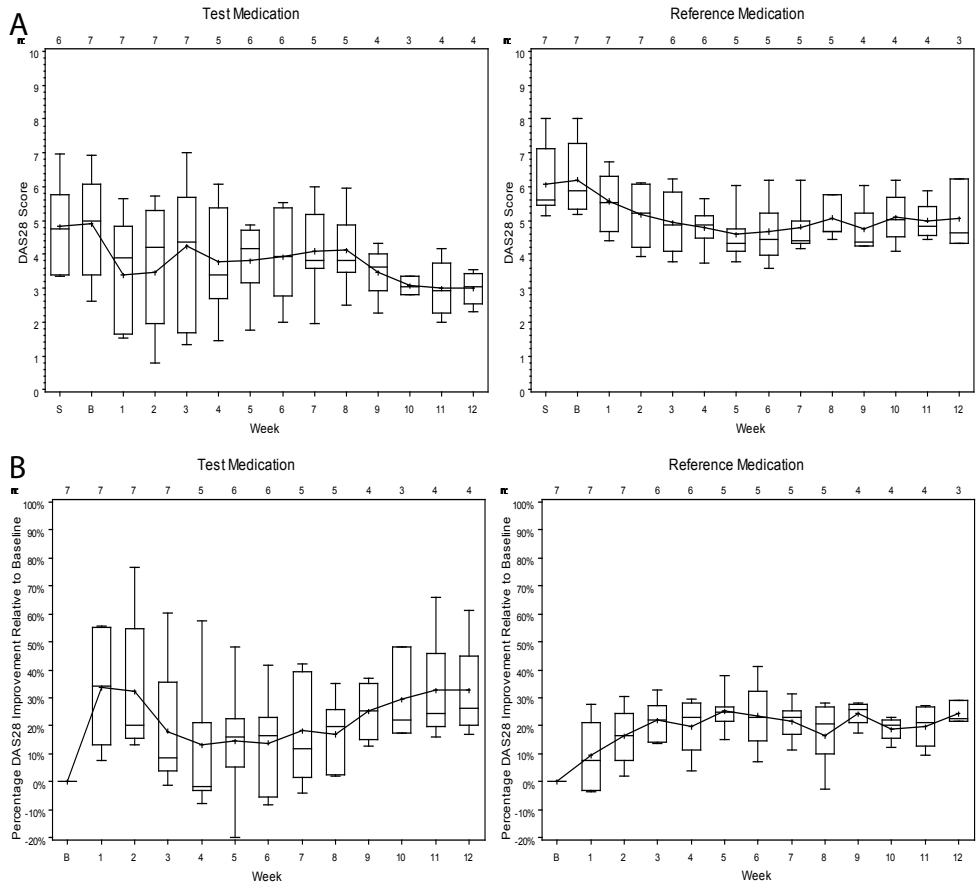

Figure 1: DAS28 Score by visit and treatment in the *per protocol* population (n=7 per treatment arm) (A), and percentage improvement of DAS28 Score by visit and treatment in the *per protocol* population (n=7 per treatment arm) (B). Curve shows mean values, rectangular bars show standard deviations split by median value. Error bars indicate 95% confidence interval. S = screening visit, B = baseline.

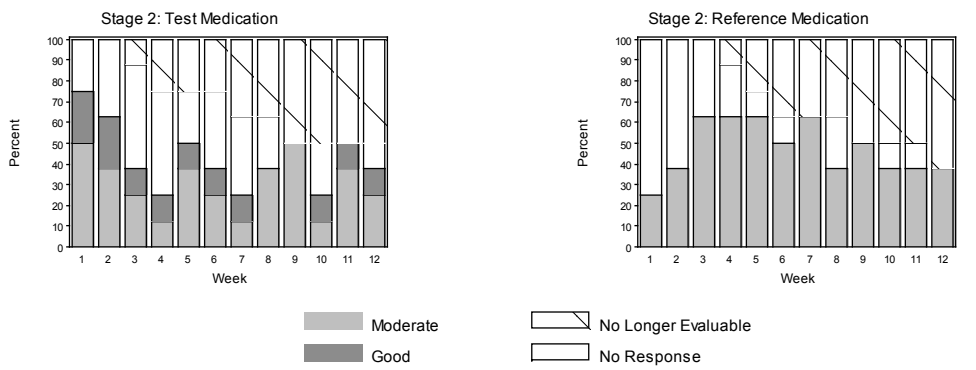

Figure 2: Bar Charts of the EULAR response in the *per protocol* population (n=7 per treatment arm).

VAS score

The intensity of pain was measured using a VAS score with a 100mm line ranging from "no pain" to "extreme pain". The pain improved better and decreased more rapidly in the LCLP group as shown in Figure 3 but the difference between the treatment groups did not reach significance.

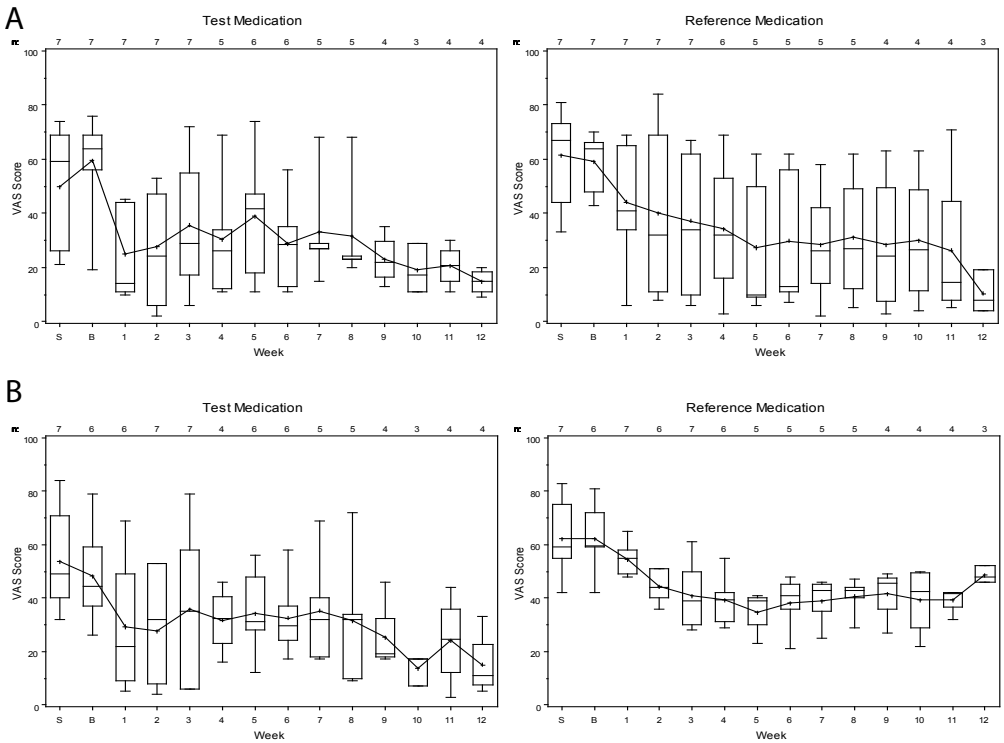

Figure 3: VAS score of pain in *per protocol* population **(**A) and VAS Score of RA activity in *per protocol* population (B). Curve shows mean values, rectangular bars show standard deviations split by median value. Error bars indicate 95% confidence interval. S = screening visit, B = baseline.

Safety

All patients completed the 12-week safety analysis which showed comparable pattern of AE's with similar distribution among organ systems in both treatment groups (Table 2). Adverse events were common but in the majority of patients they were not considered related to the medication. Four patients, all treated with LCLP, developed serious adverse events, of which only one was considered probably related to the trial medication and consisted of pyrexia immediately after LCLP infusion which recovered after administration of clemastine and acetaminophen. Blood and study drug cultures were negative. In another patient, the LCLP administration was withheld at 15 minutes after initiation because of

abdominal complaints, which were judged unrelated to the study medication by the investigator. This patient was excluded from further pharmacokinetic and efficacy analysis. At baseline, one patient was known with type II diabetes and several other patients had insulin resistance. Insulin resistance assessed by the Homeostasis Model Assessment (HOMA) and the Quantitative Insulin Sensitivity Check Index (QUICKY) did not change during the study. A short-lasting, physiological suppression of the adrenal axis was observed in both therapy groups and lasted longer in the LCLP group but recovered within two weeks. No GC-related adverse events such as weight gain, changes in lipoprotein patterns or bone-turnover markers (osteocalcin, N-Telopeptide) were observed during the study. None of the safety laboratory assessments showed clinical relevant changes.

Table 2: Adverse events

	Open label LCLP N = 6	**Trial medication N = 8**	**Control medication N = 8**	**Total N = 22**
Total adverse events, no. of patients	6/6	8/8	8/8	**22/22(100%)**
Relation to study medication				
Not related	1/6	0/8	1/8	**2/22 (9%)**
Possibly related	4/6	7/8	7/8	**18/22 (82%)**
Probably related	1/6	1/8	0/8	**2/22 (9%)**
Serious adverse events	1/6	3/8	0/8	**4/22 (18%)**
Serious adverse events probably related to study medication	0/6	1/8	0/8	**1/22 (4.5%)**
MedRA "infections &infestations"	3/6	7/8	5/8	**15/22 (68%)**
Most frequent adverse events				
Nasopharyngitis	3/6	7/8	3/8	**13/22 (59%)**
Hot flush	1/6	1/8	4/8	**6/22 (27%)**
Sleep disorder	3/6	0/3	3/8	**6/22 (27%)**
Diarrhoea	1/6	3/8	1/8	**5/22 (23%)**
Dizziness	2/6	2/8	1/8	**5/22 (23%)**
Skin ulcer	2/6	1/8	2/8	**5/22 (23%)**

Pharmacokinetics

As compared to the literature data with regard to the pharmacokinetic behavior of prednisolone when administered in free form, encapsulation in LCL results in a dramatic increase of plasma levels and half-life (Table 3) (29,30). The extremely high plasma concentration of 45 microgram/mL prednisolone phosphate after 150 mg LCLP is a direct consequence of the very small volume of distribution of the liposomes, which is not much larger than the plasma volume itself and a factor 20 smaller than the distribution of free prednisolone (29,30). Also the half life of almost three days is in line with the long-circulating properties of the LCL in humans (31). As shown by Figure 4 this results in measurable plasma concentrations of prednisolone phosphate up to six weeks after administration.

Table 3: Pharmacokinetic parameters for LCLP

	37.5 mg (n=3)	75 mg (n=3)	150 mg (n=7)
C0 (µg/mL)	16.9	28.8	44.7
AUC(0-168h) (µg/h/mL)	856	1355	4135
T1/2 (h)	45	42.7	62.5
CL (L/h)	0.041	0.054	0.031
Vss (L)	2.18	2.43	2.76
AUC(0-∞) (µg.h/mL)	957	1490	5491

It is important to note that the very high levels of prednisolone phosphate measured in blood are not pharmacologically active as the compound remains associated with the LCLP formulation here. The pharmacological effect can only be exerted once prednisolone phosphate is released and converted into the active parent drug (prednisolone) by macrophages at the target site (34). Still though, Figure 4 does show that systemic free prednisolone is detectable after LCPL administration, presumably as a result of liposomal clearance by liver macrophages and lymphoid organs (35). However, compared to the encapsulated prednisolone phosphate concentrations, the systemic exposure to free prednisolone is very low, and most marked in the first weeks after LCLP administration whereas liposomal prednisolone phosphate is measurable up to six weeks after administration. The systemic levels of free prednisolone cause suppression of the morning cortisol, indicating that the HPA-axis is not left unaffected. However, this systemic effect is short lasting and reversed within two weeks (data not shown).

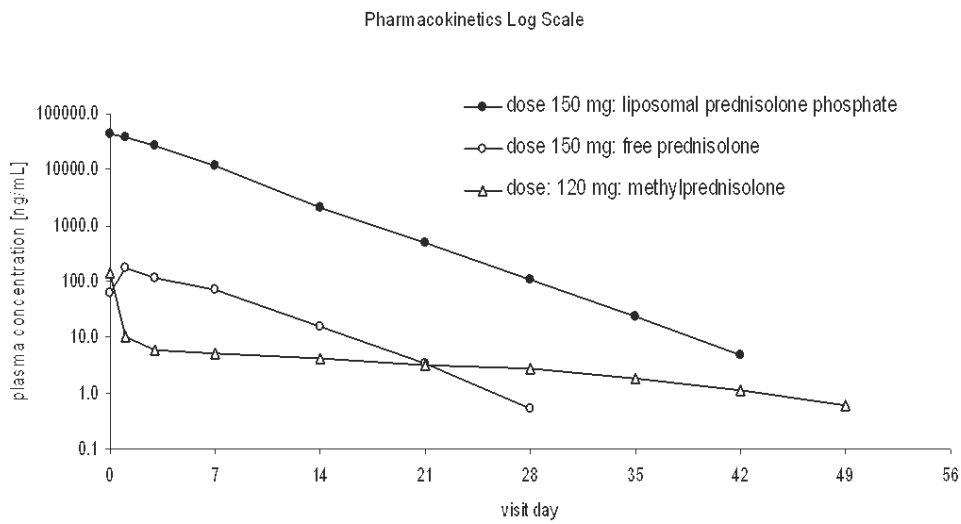

Figure 4: PK results after infusion of 150 mg LCLP and 120 mg methylprednisolone

Discussion

Despite the success of biologics of recombinant origin in RA (such as infliximab, adalimumab, abatacept, etanercept) GCs still have an role to play in the therapy of RA and many other rheumatic diseases. Early in the disease, GC pulse therapy, i.e. short-term high dose followed by medium term low-dose GCs, can accelerate the clinical response and, more importantly, improve radiologic outcomes also in the long term (34-38). Whether these disease modifying effects in early RA can also be achieved in established RA is however unclear (39). In established RA, i.m. GC, at dosages as in the present study, is rather given as bridging therapy (40-42). However, the onset of the effect is often slow and the therapeutic success of i.m. methylprednisolone varies from patient to patient as it is difficult to control the exact release rate from the intramuscular GC depot (43).

With LCLP we aim at a more effective, faster and safer GC treatment strategy. Like i.m. methylprednisolone, LCLP also employs the depot and release approach but in the case of LCLP the depots are generated selectively at the inflamed target sites. In fact, it is much comparable to the idea of intra-articular GC injections, but with the advantage of reaching all inflamed joints at the same time by one single i.v. infusion, likely generating much higher concentrations of GC at the target site with less long-term systemic exposure. The latter is also of prime importance, as the majority of the detrimental adverse effects of GC are associated with long-term systemic exposure.

The results presented by this paper support this idea. Among the most striking findings are the changes in pharmacokinetic behavior of the GC caused by liposomal encapsulation. The distribution volume was decreased with more than a factor 20 to basically the plasma volume itself and the circulation half life is increased from a few hours (regular oral/i.v. GC administration) to several days (29,30). Compared to i.m. methylprednisolone depot formulation the plasma levels have increased with roughly a factor 500. The enormous AUC increase does, however, not translate to high systemic activity, as the prednisolone phosphate measured in the circulation is tightly associated with the liposomes and systemically inactive. The assumption is, though, that the high plasma concentrations of LCLP do translate to comparable high tissue concentrations at inflamed target sites, simply because inflammation renders the local vasculature permeable and the synovial tissue directly accessible for the liposomes. In an average arthritic synovium large quantities of activated macrophages and other inflammatory synoviocytes are present, that are able to take up and digest the liposomes as soon as they have extravasated (17). The endo/lysosomal degradation of the liposomes will release incorporated prednisolone phosphate and convert it into active prednisolone that can either exert its pharmacological activity in the macrophages themselves or in other activated immune cells in the target site (32).

Despite having safety assessment as its primary objective, the study results clearly hint at the expected increased efficacy of LCLP over i.m. methylprednisolone. Although the patient population is yet too small to show statistical significance ($p = 0.07$), the DAS 28 and VAS reveal a tendency to a stronger and faster effect of LCLP during the first weeks after treatment. Also we found that Good Responders as defined by the EULAR criteria are only present in the LCLP treatment group. With respect to safety of LCLP, the study data does not raise toxicity concerns additional to the well-known systemic effects of unencapsulated GC. Adverse events occurred frequently, but only two adverse events were designated to be probably related to the trial medication. Importantly, no signs for the most feared GC adverse effects such as long-term changes in bone and blood markers were observed. Another phenomenon that deserves attention in this respect is the suppression of the HPA axis due to systemic availability of GC. As expected, treatment with i.m. methylprednisolone results in very low levels of free steroid in the circulation for a very long time (measurable up to six weeks after administration). LCLP results in higher levels but for a shorter period of time. These systemic levels translate to suppression of fasting plasma cortisol levels, which is in the case of LCLP clearly apparent during the first week after therapy but completely reversed after two weeks. This confirms the hypothesis that i.v. administration of LCLP yields prolonged presence of massive quantities of (inactive) liposomal GC in the circulation sufficient to allow the long-term generation of specific GC depots at target sites, whereas systemic adverse effects of this treatment will mostly be limited to the first weeks after treatment. Studies show that a short term systemic exposure to GC and subsequent HPA suppression is generally fairly well accepted in terms of safety (44).

Taken together the results reported in this publication indicate that GC targeted to inflamed joints by i.v. LCLP therapy may be a safe novel way to effectively intervene in RA in a single infusion or bridging approach, and that LCLP treatment may show a favorable benefit risk ratio as compared to GC standard of care. Larger studies are necessary to further investigate the added benefit, the optimal dose and the possible safety issues.

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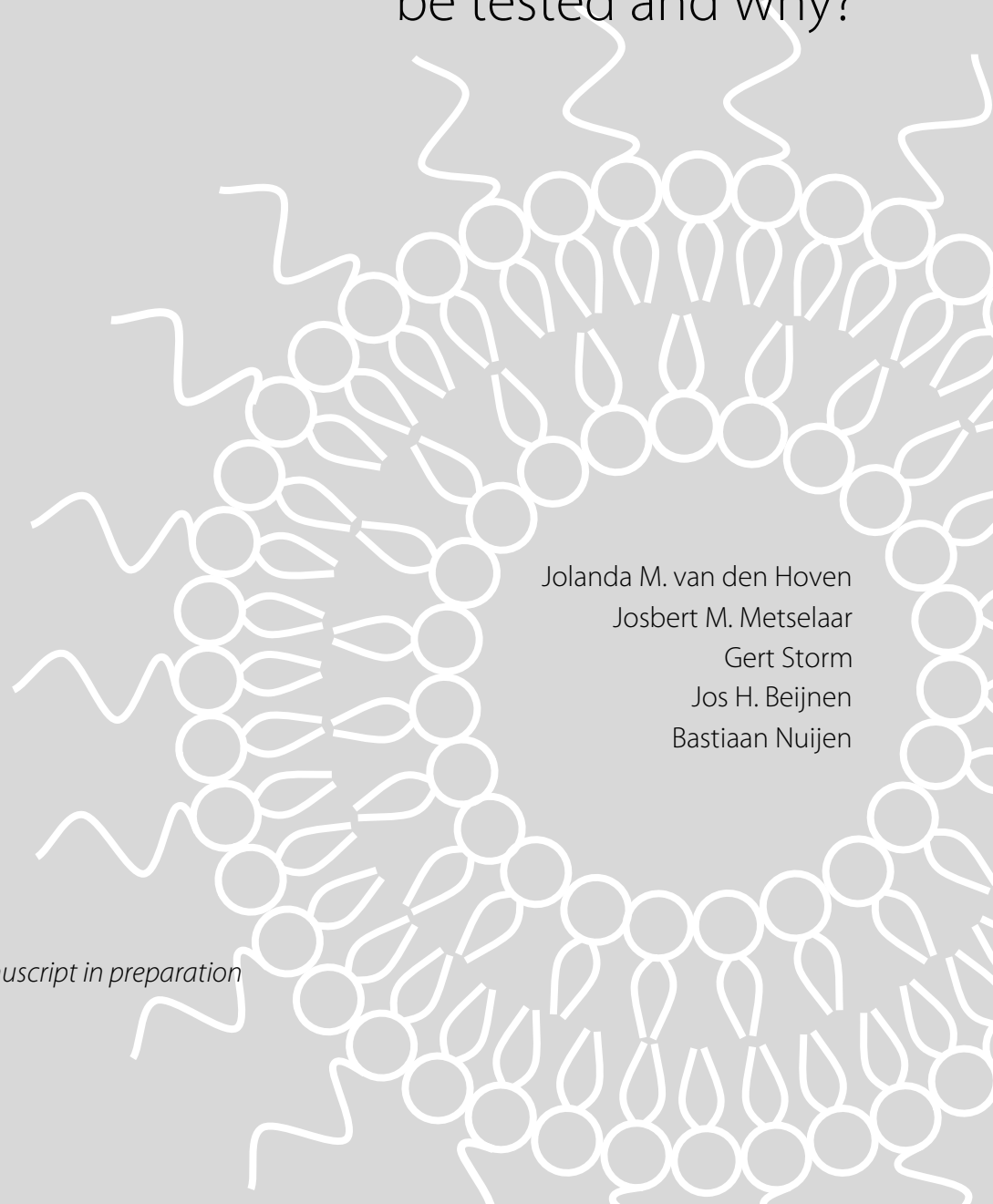
Chapter 4

Regulatory aspects on liposomal formulations. What should be tested and why?



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Abstract

The pharmacological performance of a liposomal formulation critically depends on its physicochemical characteristics. Small changes in one of these characteristics can have a huge impact on the in vivo behavior of the formulation. Therefore, the characterization of the liposomal formulation is of pivotal importance. In this article, liposomal drug product quality in relation to e.g. excipients used, manufacturing process applied and available methods of analysis are reviewed. Moreover, the current regulatory framework regarding (investigational) medicinal products and its implications for liposomal products is discussed. In this discussion, the only specific (draft) guidance document available thus far on liposomal drug product quality, as was developed by the FDA in 2002 is taken into account and recommendations and additions for more specific guidance are given. We conclude this draft guidance is a useful document for the regulation of liposomal drug products, in addition to the existing general guidelines for drug products, however, the draft document is eligible for an update to the current state of the art in liposomal physicochemical characterization techniques. More specifically, we advocate visual analysis of the liposome characteristics (e.g. by means of transmission electron microscopy) to be an integral part of the quality control of the liposome drug product, and therefore should be included in the guidance document as a standard test. In addition, standardized assays regarding liposomal safety (e.g. protein binding, complement activation) need to be included as general required test items. Additional tests regarding the product specific characteristics that are not covered by the general document should be defined on a product-by-product-basis. The updated guidance could be used in addition to existing regulatory guidelines, and would improve and simplify regulation of new as well as future generic liposomal drug products worldwide.

Introduction

In 2003 Law School Candidate John Miller published an alarming article exploring the regulatory challenges that the United States Food and Drug Administration (FDA) would encounter with respect to future nanomedical products. He foresaw problems regarding the positioning of these products in the FDA drug classification scheme and pointed at the challenge of acquiring and maintaining adequate scientific expertise in the field of nanomedicines. He advised at the time "that the FDA should start to prepare for the coming revolution in nanomedicines" (1,2). Three years later, in August 2006, the FDA launched the Nanotechnology Task Force to "determine regulatory approaches that encourage the continued development of innovative, safe, and effective FDA-regulated products that use nanotechnology materials, to identify and recommend ways to address knowledge or gaps in the nanotechnology policy and to facilitate the safe and effective use of nanoengineered materials in FDA-regulated products." (2,3). In February 2007, the Task Force concluded that the regulation of nanoproducts should be performed using a case-by-case approach utilizing the usual regulatory approval process, as was done in the early days of biotechnology products, until more specific guidelines regarding efficacy and safety would become available (2).

With respect to the continent, the European Medicines Agency (EMA) initiated the First International Workshop on Nanomedicines in 2010, with the objective to explore scientific aspects specific to nanomedicines, to share experience at an international level, and to prepare for the evaluation of future nanomedicines (4,5). As the group of nanomedicines is highly diverse and likely will become more diverse in the near future (e.g. medicines and medical devices like liposomes, polymer- and carbon-based nanomaterials, but also tissue engineering implants, nanodiagnostics and maybe even nanorobots), and classification is not clearly defined, an overall general guideline may potentially lead to "gaps" in the quality, efficacy and safety evaluation of these products. Therefore, also the EMA advocates a case-by-case assessment, until more specific guidelines are developed, based on scientific knowledge and (clinical) experience. (6-8).

Liposomes can be considered frontrunners in the field of nanomedicines. Indeed, most of the nanomedicines currently authorized by FDA and EMA are liposomal formulations (Table 1) (4,9). Besides these, many liposomal drug formulations are in various stages of clinical development, mainly for the treatment of cancer and infectious diseases. As shown in Table 1, especially liposomal products for parenteral (e.g. intravenous, intrathecal) application have proven successful. Therefore, in this article the current regulatory status of (parenteral) liposomal drug formulations as representative of nanomedicinal products is reviewed. Recommendations for improvement of liposomal quality assessment are given, which may also be applied to other classes of (future) nanomedicinal products.

Properties and performance of liposomal medicinal products

Liposomes are often used as drug delivery vehicles to increase the therapeutic index of the encapsulated drug. Liposomes can target a drug to its intended site of action, thus enhancing its therapeutic efficacy (site-specific delivery) and/or direct a drug away from those body sites that are particularly sensitive to the toxic action of it (site-avoidance delivery) (10). This was seen when doxorubicin was encapsulated in polyethyleneglycol (PEG) coated liposomes (Caelyx®, this is the EU registered name for PEGylated liposomal

Table 1: Parenteral liposomal formulations authorized by the EMA

Product	API	Formulation	Indication	Year of authorization	Licence holder
AmBisome®	Amphotericin B	Liposomes (<100 nm), API integrated in the liposomal membrane	Systemic fungal infections with *Aspergillus, Candida* or *Cryptococcus* species and visceral leishmaniasis	1990	Gilead Sciences International Limited, U.K.
Caelyx®	Doxorubicin	PEGylated liposomes (~80 nm)	Advanced ovarian cancer in women who have failed a first-line platinum-based chemotherapy regimen	1996	Janssen-Cilag International N.V., Belgium
DaunoXome®	Daunorubicin	Non-PEGylated liposomes (~45nm)	Advanced Kaposi's sarcoma associated with HIV	1996	Diatos SA, France
DepoCyte®	Cytarabine	Multivesicular lipid-based particles (~10µm)	Lymphomatous meningitis	2001	Pacira Limited, U.K.
DepoDur®	Morphine	Multivesicular liposomes (17 to 23 µm)	Pain following major surgery	2006	Flynn Pharma Limited, U.K.
Mepact®	Mifamurtide	Liposomes (<3000 nm), API integrated in the liposomal membrane	High-grade non-metastatic osteosarcoma (orphan drug)	2009	IDM PHARMA SAS, France
Myocet®	Doxorubicin	Non-PEGylated liposomes (100-230 nm)	Metastatic breast cancer	2000	Cephalon Europe, France
Visudyne®	Verteporfrin	Lyophilised powder, which is reconstituted to a liposomal solution (liposomes <100nm)	Exudative (wet) age-related macular degeneration and subfoveal choroidal neovascularisation secondary to pathological myopia.	2000	Novartis Europharm Limited, U.K.

doxorubicin that is used throughout this article, which is equivalent to the USA registered product Doxil®). Compared to conventional doxorubicin, liposomal doxorubicin showed reduced cardiotoxicity and fewer occurrences of neutropenia, anemia, alopecia, nausea and vomiting. Especially the reduced cardiotoxicity is a major advantage of Caelyx® over non-liposomal doxorubicin (11). AmBisome (liposomal amphotericin B) and DepoCyte (liposomal cytarabin) aim for similar benefits of reduced toxicity and selected target localization (12,13). Furthermore, liposomes can be useful vehicles to overcome formulation problems of the active compound as a result of for instance low solubility of the compound (14-16).

Encapsulation of an active pharmaceutical ingredient (API) into a liposome typically results in a change in its pharmacokinetic profile and its body distribution as compared to the API in a non-liposomal form given by the same route of administration (17). This can lead to differences in pharmacokinetics, pharmacodynamics and efficacy of the API, as well as to alterations in its safety and toxicity profile. All these alterations require to be investigated thoroughly (8).

Liposomes have proven to be well tolerated carrier vehicles, as most liposomes consist of (semi)natural, biodegradable lipids (18). The structure of the liposomes enable encapsulation of hydrophilic compounds in the aqueous core as well as hydrophobic compounds in the lipophilic bilayer. The physicochemical properties of the liposomal membrane can be changed to optimize drug delivery across biological barriers and drug retention at the target site, and to prevent premature degradation and toxicity to non-target tissues (19-25). Modifications at the liposomal surface could reduce the uptake of the liposomes by liver and spleen, and increases their circulation time (26,26-28).

Liposomal size is of major importance for the biodistribution of the liposomes. Passive targeting of long-circulating PEGylated liposomes, as for instance Caelyx®, depends on the enhanced permeability and retention (EPR) effect. PEGylated liposomes extravasate through the leaky microvasculature of tumor tissues or inflamed sites because of their long circulating properties and their small particle size. They accumulate in the extravascular space (often referred to as the EPR effect) (29). Changes in size would therefore change the access to target tissues of the liposomal formulation. In addition, the release profile of the encapsulated drug depends on the morphology and lamellarity of the liposomal membrane, and on its thickness, permeability and rigidity (30-34). The permeability is regulated by the phase transition of the lipid membrane. Below this phase transition temperature (T_m), liposomes have low permeability to the encapsulated molecules (unless the molecule is membrane permeable). At a given temperature a lipid in the lipid bilayer can exist in the tightly packed gel-like phase or the more fluid and dynamic liquid-crystalline phase. When the temperature is increased, a gel-like bilayer becomes liquid-crystalline, and this increases the membrane permeability. Addition of cholesterol makes the liposomal membrane less permeable in the liquid-crystaline state (34,35).

A schematic overview of possible variations in liposomal characteristics is given in Figure 1.

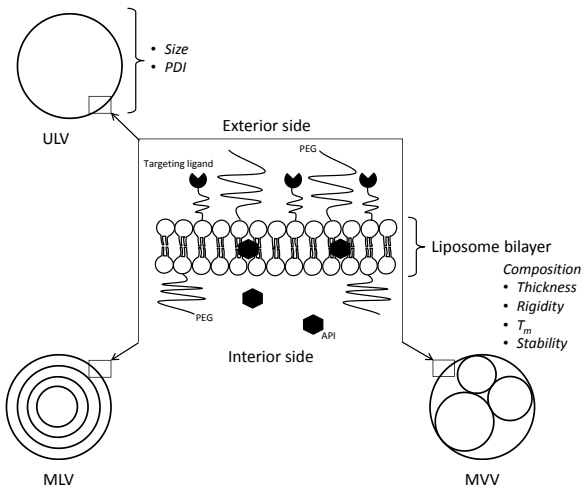

Figure 1: Schematic overview of the possible variations in liposomal characteristics. Abbreviations: ULV, uni-lamellar vesicle; MLV, multilamellar vesicle; MVV, multivesicular vesicle; API, active pharmaceutical in-gredient; PEG, polyethylene glycol; PDI, poly dispersity index; T_m, phase transition temperature of the membrane.

The performance of a liposomal formulation is critically dependent on the liposomal characteristics. This can be illustrated by liposomal doxorubicin, of which 2 formulations are marketed: a PEGylated formulation (Caelyx®) and a non-PEGylated formulation (Myocet®). This results in major differences in their pharmacokinetic parameters, as can be seen from Table 2.

Table 2: Pharmacokinetic parameters for Caelyx®, Myocet® and non-liposomal doxorubicin (36,37).

	Caelyx®	**Myocet®**	**Non-liposomal doxorubicin**
Volume of distribution (L)	3.33	5.1	46.7
Clearance (L/h)	0.05	56.6	1451

However, liposomal formulations with the same lipid composition can also differ with regard to for instance morphology, size, size distribution and charge, even from batch to batch. Typically, small changes in one of these parameters can have a huge impact on the in vivo behavior of the formulation, resulting in changes in the therapeutic efficacy or the toxicity of a formulation (31), as was shown for liposomal doxorubicin by Mayer et al. (38). To put it differently: a small change in one of the liposomal characteristics could make their in vivo behavior unpredictable (2,39-41), and potentially hazardous (6,7,42,43). Therefore, a regulatory documentation structure with requirements for the registration and approval of liposomal drug products is mandatory.

Current regulatory guidelines regarding liposomal drug formulations.

Liposomal medicinal products have been authorized for human use worldwide. In none of the countries or regions where registration has been pursued, specific guidelines for liposomal drug products as a group have been approved. Like other drugs, more general regulatory guidelines for pharmaceutical products are laid down in harmonized (EU, US, Japan) guidelines by the "International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use" (ICH) (44,45). These guidelines address key assessments with respect to quality, efficacy, and safety required for the successful development and registration of a pharmaceutical product for human use (46). With respect to the manufacture of (investigational) medicinal products, Good Manufacturing Practices (GMP) guidelines apply (47-49). Currently, only a draft guideline on the submission and registration of liposomal drug products exists (50). This document provides recommendations with respect to chemistry, manufacturing and controls, human pharmacokinetics and bioavailability, and labeling of liposomal drug products, focusing on the unique technical aspects of these formulations. No guidance is given with respect to e.g. clinical safety and efficacy or bioequivalence. To start with, the document, rightly, highlights the importance of characterization. Characterization of the physicochemical properties, e.g. morphology, charge, size, phase transition temperature and in vitro release, are of importance for quality control, but can also be beneficial in evaluating changes in the manufacturing protocol. Second, the document advises on the extent of human pharmacokinetics and bioavailability studies (both in vitro and in vivo) that are required when submitting a new drug application for a liposomal drug product. A (bio)analytical method suitable to determine both the encapsulated and unencapsulated fraction of the drug can be used in pharmacokinetic analysis as well as in quality control. And finally, the document refers at some product labeling issues for (new) liposomal drug formulations.

Most of the currently authorized liposomal formulations are novel formulations of existing, previously marketed non-liposomal APIs, developed in order to more specifically target the disease and/or reduce adverse events. These new liposomal formulations were approved based on at least part of the safety and efficacy data used to register the non-liposomal API (39,42,51). However, considering the significant changes in the pharmacokinetic and pharmacodynamic profiles, the efficacy and safety of a liposomal formulation should be judged on its own merits (41,42).

Up to this date no generic liposomal formulations have been approved for clinical use. In the near future liposomal doxorubicin (Caelyx®) will become available as a generic formulation. The patent of Caelyx® has expired already in 2009, and the first application for registration of a generic liposomal formulation containing doxorubicin has already been reviewed by the EMA (52). An important aspect is the assessment of bioequivalence of these generic

formulations to the registered formulation. To demonstrate bioequivalence of liposomal formulations containing doxorubicin, the FDA already published a draft guidance, in which they state that the registered liposomal formulation should be used as a reference standard in all analyses (53).

Important test items in the design and development of liposomal drug formulations

In the first place, liposomes are designed to fit their intended purpose. Characteristics (e.g. size, lamellarity, surface charge and PEGylation profile) are selected based on the target organ and the route of administration. Based on these characteristics, the pharmacokinetic-, biodistribution- and excretion profiles of the liposomal drug product can be predicted to a large extent (17,50). These characteristics are directly related to the liposomal composition (type of lipids and their ratio) and the physicochemical properties of the API. During the design phase, the critical attributes must be identified and the analytical tests for qualification or quantification of these characteristics must developed, validated and implemented (4). The critical characteristics, the acceptable limits, and related process parameters will make up the 'design space' of the drug product. In the following paragraph, a range of possible important test items and analytical methods for liposomal drug formulations will be discussed.

During further development of the liposomal drug product, the formulation and the production process may be refined and improved based on updated product and process understanding. Sources of variability that have an impact on the quality of the final product are identified, understood and controlled, to deliver a constant drug product quality. Based on this knowledge, critical product attributes are to be selected and included in the quality control of the final product. This means that extensive characterization of the liposomal formulation already needs to be initiated early in the development of the formulation, and implies the need for analytical methods to asses these liposomal characteristics.

Formulation components: Raw materials

As mentioned, the liposomal drug formulations have to comply with the ICH guidelines. Like for all drug formulations, both API and all excipients have to be pure and safe. For liposomal drug products, special attention should be paid to the lipid excipients used, since these form the liposomal membrane and thereby are key for the biodistribution profile and the pharmacologic performance of the API. Small changes in quality and purity of lipid compounds can have a major effect on the quality and safety of the final liposomal drug formulation. Small amounts of free fatty acids, lysophosphatides or other lipid degradation products can have significant effects on the surface charge, size and permeability properties of liposomes (54-56). This highlights the importance of extensive characterization of the lipid

compounds (50,57)(50,57)(50,54), the degree of characterization depending on the nature of the compound. This extensive characterization of the lipid excipients is adequately described in the FDA draft guidance (50,57). Source, raw materials, production process (including process controls (IPCs)) and manufacturer need to be described. Animal derived lipid products and semi-synthetic lipids should be guaranteed to be free of viral or protein contaminations (like for instance BSE). Purity of each lipid batch should be assessed using a stability indicating assay, as compared to a reference standard (if available) (50). In addition, the lipid compounds should undergo stability studies and stress testing, to determine their storage conditions, shelf life and degradation profile. Impurities and degradation products (like stereochemical impurtities and isomeric forms, but also degradation products due to oxidation or hydrolysis of the lipid compounds (58,59)) should be determined and analyzed (50).

Production process: Validation and In Process Controls (IPC)

Manufacturing of a liposomal product has to meet the GMP quality standards. Amongst aspects like personnel, facilities and documentation, this involves the definition and control of critical process steps (steps that determine the characteristics of the final liposomal formulation). Figure 2 shows a flow chart of a general liposomal manufacturing process. The process starts off with the preparation of a coarse dispersion. The API of interest can be directly mixed with the lipids, resulting in passively loaded liposomes, or can be actively loaded into empty liposomes after the sizing step. Liposomes have to be down-sized at temperatures above their T_m, using methods that cause extensive mechanical stress and shear (e.g. extrusion, high shear homogenization or high pressure techniques).

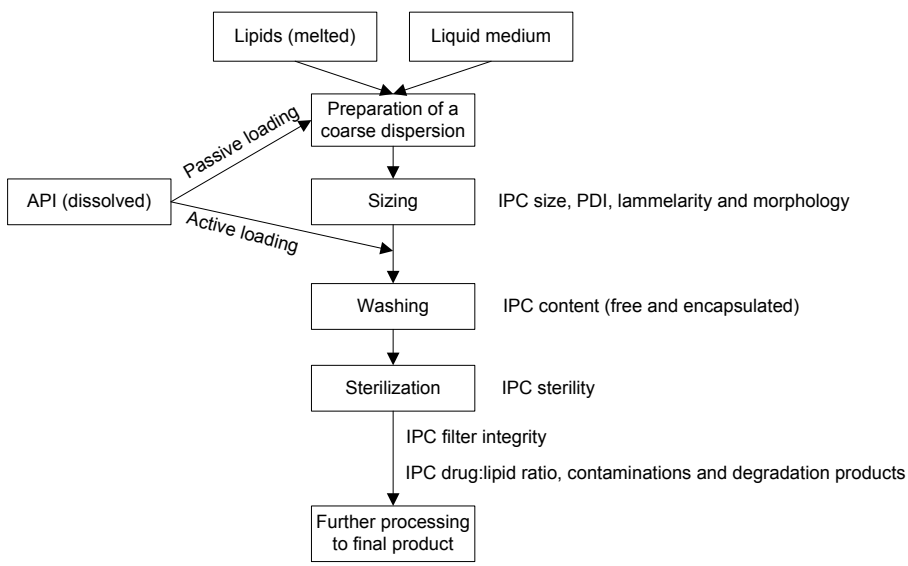

Figure 2: Process flow sheet for the production of (sterile) liposomal drug products. Abbreviations: API, active pharmaceutical ingredient; IPC, in process control; PDI, poly dispersity index.

Additionally, the external API and residual organic solvents (if applicable) need to be washed away, which can be performed using tangential flow filtration or dialysis. Then the liposomal formulation needs to be further processed into a final product. From this it is easily understood that the manufacture of liposomes is a complex process. In particular the method of liposome formation, inclusion of API and subsequent sizing are critical process steps which are sensitive for small changes in the manufacturing conditions (50). Also, methods or equipment used are not per definition scalable and interchangeable. This all poses manufacturers for significant challenges to identify, set specifications and control critical process steps to comply with GMP quality standards (60,61).

The production process needs to be robust and the effect of factors like e.g. mixing speed, buffer or solvent concentrations, temperature, pressure, equipment and personnel on the quality of the final product has to be validated, because of the complexity of both the process and the final product (50). Determination of the critical steps in the liposomal production process and controlling these steps by in process sampling and adequate assays is extremely important to guarantee the quality of the final product (50). In process controls can for instance involve the analysis of degradation products and other possible contaminations, or to check the size and size distribution of the product during the production process. By measuring in process controls the production process is monitored and can be adapted if necessary, in order to obtain a final product of the desired quality. The manufacturers should judge by themselves whenever they have gained sufficient evidence to assure that the production process is capable of consistently delivering products of the desired quality (62).

Also changing the scale of the production process, e.g. from clinical trial batch size to a commercial production setting, could have a major influence on the resulting liposomal drug product. Therefore, in process control sampling and complete characterization of the resulting liposomal drug product is important when the production scale has been changed. In vivo studies may be warranted if this characterization indicates some change in the performance characteristics of the liposomal drug product. (50,60). The recent manufacturing issues and the subsequent delay in transfer of the commercial production of Caelyx® to another plant exemplifies that the transfer and implementation of a GMP-compliant manufacturing process of a liposomal product is not a straightforward plug-and-play (63,64).

Characterization of the liposomal drug product

For the final liposomal product extensive characterization of the physicochemical properties, is recommended (5,17,50,57). Test items are summarized in Table 2, and the specific analytical techniques are summarized in Table 3. The importance of some of the characteristics will be discussed in the following paragraphs.

Table 2: Test items for the characterization of liposomal drug formulations

Liposomal components • Purity API • Crystallinity API • Encapsulated API (including salt forms) • Unencapsulated API • Lipid contents / Lipid composition (ratio) • Degradation products of the lipid components • Drug to lipid ratio • Related substances API and lipid components • Buffer composition	**Liposome characteristics** • Size • Size distribution • Presence of aggregates • Internal volume • Biological activity • Immunochemical properties and interactions • In vitro (plasma) stability and release • Long term toxicity profile
Bilayer characteristics • Morphology • Lamellarity • Surface area • Conformation of surface modifying compounds • Charge • Thickness membrane layer • Phase transition temperature	**Liposomal dispersion** • Appearance • Residual solvents • Uniformity of dosage units • pH • Sterility / Microbial limits • Bacterial endotoxins • Particulate matter • Water content • Reconstitution time • Antimicrobial and antioxidant preservative content • Osmolality • Extractables • Stability upon storage

Liposomal components

Qualitative analysis and quantification of the components of the liposomal formulation are important items to start with and include both the lipid components and the enclosed API. When a drug is enclosed in the liposome in a different salt form or crystal form, as for instance the crystal structures ((doxorubicin)$_2$SO$_4$-salt) of the remote loaded doxorubicin in Caelyx®, both forms have to be quantified. Of particular importance is the ratio of the separate lipid components of the liposome, since a different ratio could result in a liposome with completely different properties, changing the biodistribution- and pharmacokinetic profile of the formulation. Therefore, quantification of the liposomal compounds is of major importance for both safety and efficacy of the drug product (17,50).Degradation products of lipid components or the enclosed drug that were not present in the raw materials could be an indication that the liposomal formulation is chemically unstable. Levels of free API have to be quantified as it is indicative for either physically unstable liposomes, resulting in drug leakage, or an insufficient washing step during manufacture. Presence of free drug or degradation products above threshold limits can cause changes in pharmacokinetics and/or result in toxicity.

Table 3: Analytical techniques used for the characterization of liposomal drug formulations.

Analytical technique	Liposomal property	Liposomal property has an influence on:	Characteristics of	Ref
HPLC (UV, MS detection), or other suitable assay method	Content and purity API	Efficacy and toxicity	Liposomal components	(57,65)
HPLC (UV, MS, ELSD detection)	Content and purity lipid components	Efficacy and toxicity, liposomal properties, release profile of the encapsulated drug	Liposomal components	(38,38, 65,66)
	Type of surface modifying compounds and their 3D conformation (indicative)	Circulation time, clearance, distribution, cellular uptake	Liposomal bilayer	(26)
	Thickness of the liposomal bilayer (indicative)	Aggregation in the formulation and interactions with proteins and cells in vivo	Liposomal bilayer	(67-69)
Small angle scattering	Thickness of the liposomal bilayer	Aggregation in the formulation and interactions with proteins and cells in vivo	Liposomal bilayer	(70)
Zeta potential measurement	Surface charge	Interaction and uptake by target cells and MPS, toxicity by rupture of cell membranes	Liposomal bilayer	(8,65, 71-73)
TEM	Lamelarity, morphology and thickness of the liposomal bilayer	Release profile of the encapsulated drug	Liposomal bilayer	(30-33)
	Drug crystallinity	Correct salt form, shape of the precipitate	Liposomal components	(33, 74-76)
DSC	Phase transition temperature	Permeability of the liposomal membrane, release profile of the encapsulated drug	Liposomal bilayer	(30,65, 77)
DLS, Nanosight, SEC, FFF or TEM	Size	Biological interactions, biodistribution	Liposomal particles	(32,65, 78-80)
	Size distribution	Indicates absence of aggregates or agglomerates	Liposomal particles	(32, 78-80)
	Surface area (indicative, related to size)	Interactions with cells, tissues, organ systems, proteins and other macromolecules	Liposomal bilayer	(6,81)
	Internal volume	Drug content (indicative) and release profile (indicative)	Liposomal particles	(82)
FACS	Detection of aggregates, even when very low numbers of aggregates are present	Aggregates could activate complement, resulting in hypersensitivity reactions, influencing the safety of the formulation.	Liposomal particles	(83)
Entrapment fluorescent probe	Internal volume	Drug content (indicative) and release profile (indicative)	Liposomal particles	(84)
Complement assay	Immunochemical properties	Complement induced hypersensitivity reactions	Liposomal particles	(27,75, 85)
Release testing method	In vitro release and stability	Release profile of the encapsulated drug, membrane stability in vivo (indicative)	Liposomal particles	(86-88)

Abbreviations: MS, mass spectrometry; ELSD, evaporative light scattering detection; TEM, transmission electron microscopy; DSC, differential scanning calorimetry; DLS, dynamic light scattering; SEC, size exclusion chromatography; FFF, field-flow fractionation; FACS, flow cytometric analysis.

Bilayer characteristics

The liposomal surface is of importance for the in vivo performance of the liposomal product. As the physicochemical surface properties of the lipid bilayer are of importance for the interactions with cells, tissues, organ systems, proteins and other macromolecules in the body, they determine kinetics, tissue distribution and cellular uptake (6,81). The surface area can be extensively modified, for instance by the addition of PEG. PEG delays liposomes from being recognized by the mononuclear phagocyte system (MPS), making them circulate for a longer period of time (26). Addition of surface structures could influence the surface charge of the liposomal membrane. High positive surface charges might disrupt cell membranes and thereby can cause side effects (8,71). The type of surface structure and its three-dimensional conformation could influence potential liposome-liposome aggregation in the formulation and might change the in vivo behaviour (67-69).

Cholesterol is often used in liposomal formulations to make the liposomal membrane more rigid, to improve the liposomal stability. Cholesterol also reduces the permeability of the liposomal membrane in the fluid liquid-crystaline state (34,35). Changes in cholesterol content therefore might change the release profile of the encapsulated drug, and needs to be quantified.

Liposome characteristics

Small-sized liposomes (<100 nm) are nanoparticles. The fact that the name features "nano" prominently leads to the thought that size is the most important physicochemical parameter. First, size affects the biological interactions, and thereby the activity of a product, profoundly changing the biopharmaceutical properties of the API given as a free, unencapsulated agent. A small change in size has a large impact on the surface area and thereby can change the biological interactions and the activity of the liposomal drug product for better or worse (6,31). Second, size is crucial for the biodistribution of the liposomal formulation, and thereby important for the liposome to reach the target site (34,38,89,90). As can be seen from Table 3, there are various techniques to determine the size of a liposome. However, different methods produce different results for the same liposomes, because they all measure a different "type" of diameter, that can be based on volume, area, number, Brownian motion (or a combination of these factors) of the liposomes. To compare the results, samples should be measured using the same method. For liposomes, the common method to determine size and size distribution is Dynamic Light Scattering (DLS). The method is fast, but the method is also easily disturbed by a few dust particles or aggregates. Precise and accurate results are only obtained for formulations having a narrow size distribution. (32,78-80).

It is known that liposomes can form supravesicular structures, such as aggregates and fused liposomal complexes. These structures can activate the complement system, even at very

low amounts present (75,83). These low numbers are often not detected by techniques commonly used for size determination, like DLS or cryo-transmission electron microscopy (cryo-TEM). Milosevits et al. developed a flow cytometric analysis (FACS) method to detect these structures even when present in an extremely low (millionth) fraction of the particles. The method could be further developed as a quality assay for liposomal homogeneity (83).

In addition to size and size distribution, the drug release profile and liposomal stability upon administration have to be determined using several in vitro tests. There is a need for a validated method for in vitro release testing of controlled release parenteral drug formulations. Rawat et al. used the USP apparatus 4 method and developed a release testing method than can be used for liposomal formulations. The conventional adapter was replaced by an adapter containing a dialysis membrane, that can be used both in the open and closed setting. Using this adapter, the release profile of liposomal formulations and other dispersed systems could be determined. The system has already been validated for release testing of microspheres and has proven to be able to discriminate between the release profile of large multilamellar vesicles and small unilamellar vesicles (86,87). To speed up the in vitro testing processes, Hoiko et al also developed an alternative in vitro stability and release assay using higher temperatures and various media (88).

Complex interactions could exist between liposomes and the environment (81). Interactions could cause problems in toxicity and safety when liposomes for instance interact with proteins, enzymatic systems or cellular pump systems (50). The interactions with for instance plasma proteins should be assessed for every liposomal drug product over the expected therapeutic concentration range. Additionally, tests for long term toxicity should also be performed, since some materials can accumulate in the body, be cytotoxic or genotoxic, teratogenic, immunotoxic, resulting in long term effects (7).

It is known that injection of PEGylated liposomes, but also larger liposomes without PEG or liposomes with alternative surface structures, can give infusion reactions (27,91,92). It is thought that this is caused by activation of a part of the innate immune system, known as the complement system. Most of these complement-induced hypersensitivity reactions are transient and mild, but life threatening reactions can occur in hypersensitive patients (75,93-98). Therefore, it is important to test the formulation for the possible occurrence of complement activation. A variety of tests are available to test the liposomes for this purpose. They vary between in vitro assays for different complement factors, to an in vivo test for hypersensitivity in pigs (27,75,85).

Liposomal dispersion

Finally, the (new) liposomal formulation should be tested according to the regional valid version of the pharmacopeia and ICH Guideline Q6A (57). The test parameters are summarized in Table 2. This includes the analysis of a potential interaction with the

container/closure system, since the extractables from the container/closure system can cause toxicity. Extractable levels should be well below the levels that are considered to be safe, and the stability of the formulation should not be affected by these extractables (57).

The stability of the final liposomal formulation upon storage has to be determined. Therefore, the stability of both the drug and the lipid compounds, as well as the stability of the liposomal membrane need to be tested on a regular basis (50). This information will be available from development and validation batches. Acquisition of stability data could be ongoing, and these data can be added after approval (57).

Quality control of liposomal drug products

The most important part of quality control of liposomal drug products is the physicochemical characterization of every new batch of the formulation. The following quality-control assays should therefore be applied (57,65,99):

- Chemical characterization and stability: identity, concentration and purity of API; identity, concentration and purity of the lipid compounds; drug:lipid ratio; encapsulation efficiency; uniformity of dosage units; pH; osmolality; particulate matter and residual solvents.
- Physical characterization: appearance; size and size distribution; trapped volume; zeta potential; phase transition temperature; lamellarity and morphology and percentage of free drug.
- Microbiological assays: sterility; endotoxin/pyrogen level.

During development, critical product characteristics can be identified. These characteristics need to be included in the quality control of the final product, to guarantee the desired final product quality. When considered critical, as judged by the manufacturer or the reviewer of the application report, in vivo tests to assure bioequivalence of the new batch might have to be included in quality control testing on a product-by-product basis. To avoid in vivo testing, representative in vitro tests could be developed to replace these in vivo tests.

Generic liposomal formulations

Generic liposomal formulations will become available in the near future. One of the first generic liposomal products will be liposomal doxorubicin (Caelyx®). In anticipation, both agencies have recently published (draft) guidance documents that describe how bioequivalence of the generic formulation (or abbreviated new drug application (ANDA)) and the registered product (or reference listed drug (RLD)) has to be established (53,100). In these documents distinction has been made between pharmaceutical equivalence (e.g. equivalence in composition, manufacturing processes and physicochemical characterization assays) and clinical equivalence (e.g. equivalence in biodistribution, in vitro or in vivo

performance and safety). In this paragraph we will describe when two formulations can be considered bioequivalent according to these documents.

First of all, the composition of the generic product must be qualitatively and quantitatively the same, except for differences in buffers, preservatives and antioxidants. The manufacturers have to prove that these differences do not have an influence on the safety and efficacy of the formulation, though the FDA does not have any recommendations for the type of studies to proof this (53). Lipids are critical components in liposomal formulations. Therefore, they should meet the specifications of the lipid components used in the RLD. Lipids should have the same category of synthesis route (natural or synthetic) as the lipid components used in the RLD, and information about chemistry, manufacturing and control should be provided at the same level of detail (53).

Second, the production processes of the liposomal formulations should be comparable. This means a comparable drug loading method in the case of doxorubicin liposomes. During development, the manufacturers should define critical parameters during the production process that should be measured as a part of bioequivalence assessment (53). Since the exact production process might not be available for the developer of generic liposomal formulations, the EMA suggests that if comparative stress testing of the formulations results in equal degradation profiles and physicochemical performance characteristics, this could be an indication for equivalence (100).

When product composition and the manufacturing method are similar, this should result in liposomes containing doxorubicin with similar liposome characteristics. This similarity has to be confirmed. The draft guidance recommends that 3 batches should be characterized on the following items:

- Chemical composition: lipid content, concentrations of free drug and encapsulated drug, including the state of the encapsulated drug
- Surface chemistry: liposomal morphology and lamellarity, phase transition of the bilayer, liposomal size, surface charge, thickness of the PEG layer and PEG concentration at the liposomal surface
- Liposome characteristics: internal environment (pH, volume, salt concentrations), size distribution and in vitro leakage.

These characteristics are considered to be the most important ones for Caelyx® and its generic products, and these tests might be product-specific. Therefore, these tests to determine equivalence should be defined on a product-by-product basis (100). It should also be defined when the test results are considered equivalent, and how much of these results might deviate from the test result of the registered product (53).

Based on the analytical tests described above two liposomal formulations might be similar in their characteristics, but the equivalence in biological activity and safety still needs to be demonstrated (100). Therefore, it is advised to test bioequivalence in various in vivo studies,

which is currently practice with generic drug products from biotechnological origin. The type of in vitro and in vivo studies should be selected on a case-by-case basis, and could include both non-clinical and clinical studies. (100). For generic liposomal doxorubicin, a two-way crossover bioequivalence study in ovarian cancer patients is recommended by the FDA, to demonstrate in vivo pharmacokinetics and stability of the generic liposomal formulation. Since both treatments are considered equal, this test is regarded safe and patients are not exposed to undue risk (53).

Similar composition, manufacturing process and characteristics are not enough to prove bioequivalence, according to the FDA and the EMA (53,100). Only when (non-)clinical in vivo studies in suitable (animal) models show equal tissue distribution, pharmacokinetics, elimination profiles, pharmacodynamic responses and toxicity, the two formulations are defined as bioequivalent (53,100). The need of these studies is reflected in the CHMP assessment paper on generic liposomal doxorubicin. Although the liposomal doxorubicin formulation is considered similar to Caelyx® regarding its characteristics, the formulations seem to have a different tissue distribution profile, release profile and pharmacokinetic profile (e.g. a significant difference in AUC of free (unencapsulated) doxorubicin over the first 48 hours), and based on these data are considered non-bioequivalent by the FDA (52). The report also reflects the need of regulatory advises regarding these non-clinical studies, since there were major concerns regarding the reliability of the data, the adequacy of the studies performed and the power of the studies performed (52). Remarkably, due to the shortage of doxorubicin hydrochloride liposomal injection (Doxil®) on the USA market, the FDA approved Lipodox® (Sun Pharma Global FZE's doxorubicin hydrochloride liposome injection, the product assessed by the EMA) temporary to the marked, though they explicitly mention that the formulation cannot be regarded a generic formulation of Doxil® (101).

Evaluation of the current guidance on liposome drug products.

The FDA draft guidance on liposome drug products

Liposomal products need to be characterized using specific liposomal product assays that address important physicochemical properties such as the surface characteristics and vesicle structure. Specific assays for these items are, however, not provided by standard medicinal drug product guidelines. In the USA, the authorities have therefore chosen to develop a draft guidance on liposome drug products that covers a range of critical test items described in this article (50). However, this FDA draft guidance was issued in 2002, and as such, is referring to the state of the art of that time. Indeed, with the liposomal field evolving and expanding rapidly, the draft guidance is eligible for an update. Also, current and future applications for generic liposomal drug formulations strongly warrants established guidance which can be integrated in this document.

Current gaps in the list of characterization assays provided by the guidance

The FDA draft guidance rightfully states that a detailed evaluation of liposomal physicochemical properties is key, as the drug product performance is critically dependent on these properties. A list of basic properties to assess is suggested in this guidance, however, with the current knowledge and expertise this list appears incomplete. The most important assay lacking is the visualization of the liposome dispersion e.g. by means of cryo-TEM. The need for visual inspection of liposomal formulations is illustrated by Dicko et al., who found that their liposomes contained a second lamella (bilamellar liposome) that was of major importance for the drug release profile of their liposomal formulation. The first indications for this second lamella were seen using cryo-TEM. To be able to fully characterize this structure, other techniques had to be used, e.g. fluorescent labeling studies and extensive spectroscopic studies (30). After confirmation of this second lamella, TEM needs to be included in the quality control program of every batch of this particular product, to confirm its presence. A second example that illustrates the need of visual inspection of the liposomal formulation is given by the unique coffee bean or American Football-like prolate spheroid appearance of Caelyx®, that can be visualized using cryo-TEM. It is widely documented that this typical shape is caused by the high concentration of elongated crystals of doxorubicin in the liposomes that form during the remote loading process (74-76). This crystal formation appears important for the plasma stability and drug release profile of the formulation (76,102), and therefore can be considered as an important quality attribute of Caelyx®. It is remarkable that this test is not included in the drug product specifications of Caelyx®, and is also not specifically mentioned as an important liposomal characteristic in the FDA draft guidance on generic liposomal doxorubicin, or in the assessment report on the first generic liposomal doxorubicin formulation (52,53). Characterization of the liposomal interior by TEM might be important for other new remote-loaded liposomal formulations in which high intraliposomal concentrations of active compounds are achieved. In addition, visualization of the liposomal dispersion gives important, at least qualitative, additive information on key structural properties like morphology, lamellarity, thickness of the membrane layer(s), shape and size. Therefore, we advocate visual analysis (e.g. by means of TEM) to be an integral part of the quality control of a liposomal drug product, and should be included in the guidance as a standard test.

We also believe that the guidance should provide the industry with standardized assays for each liposomal property. We suggest that DLS should be integrated as the standard method for assessing particle size and size distribution, while differential scanning calorimetry (DSC) should be included as the standard method to assess the phase transition temperature of the liposomal membrane. These methods are accurate, straightforward, fast and moreover widely established. In addition, a standardized, validated method for drug release testing is strongly required, like for instance the release assay based on USP apparatus 4 as developed

by Rawat et al. to determine the release profile of liposomal formulations and other dispersed systems (86,87).

Product-specific properties: whether or not to include in the guidance.

Liposomal formulations that are under development often have novel properties and modalities that require additional or more adequate characterization assays. Some of these may be highly specific for just one liposomal product which goes beyond the scope of one liposomal guidance for all liposome drug products. Analysis of the structural conformation and biological activity of for instance antibody-targeted immunoliposomes or specific ligand-targeted liposomes are examples of tests that require more specific techniques that would not be implemented in an overall document in the regulation of liposomal formulations, though these tests needs to be performed on every batch of this particular product. A case-by-case approach is advised for these highly specific assays.

Other properties and modalities may, however, be shared by a larger group of new liposomal products, e.g. PEGylated long-circulating liposomes. Specific tests to assess the conformation of the PEG chain conformation using small angle neutron spectroscopy (SANS) could be included (103). In addition, specific PEG-related protein binding assays might be developed and included in the guidance. Standardized, group specific assays can also be expected to be developed for liposomal vaccine products, liposomal products containing specific nucleic acids, stimuli-sensitive membrane components or magnetic particles. Specific assays to quantify the presence of these components in the liposomal membrane or the aqueous core, or assays to determine their structural orientation in the liposomal membrane might be required. Addition of these assays to address these group-specific items to the guidance would be valuable, because it would standardize the characterization of these properties and prevents that every manufacturer has to develop new assays for this group specific property. Addition of these standardized group-specific assays would therefore simplify the regulation of these liposome types both from the perspective of the authorities as well as the manufacturers.

The same procedure is advised for bioequivalence assessments for generic liposomal formulations. To date, only one generic liposomal formulation has been developed, and the (in vivo) bioequivalence tests selected for this product are product-specific. Evolution of this field of generic liposomal formulations will point out the possibilities regarding standardized bioequivalence assessments of new generic formulations, and the value of inclusion of these tests in the guidance document.

Safety assays of liposomal drug products that should be included in the guidance

Currently, nano-material safety is a hot topic, though no assays are mentioned in the guidance that focus specifically on the potential safety-issues with the liposomal carrier. Liposomal particle size, shape, vesicle composition, physicochemical properties of surface structures alone or in combination can lead to a range of biologic effects and interactions that raise toxicity concerns (44). Protein binding of liposomes could for instance result in increased membrane permeability and even dose dumping of the enclosed drug, altered circulation time of the liposomes, reduced or enhanced clearance rate and changes in the uptake by the mononuclear phagocyte system (MPS) (50,104,105).

Understanding which (combination of) properties interact with particular biological systems and functions is needed to guarantee the safety of liposomes and other nano-materials (7). Therefore the protein binding (which is already mentioned in the draft guidance) biodistribution and accumulation over the expected therapeutic concentration range of the liposomal carrier vehicles and the liposome membrane components in the body have to be investigated, using standardized in vivo or in vitro tests. These tests should be included in the guidance, since they might apply for every liposomal formulation. To avoid problems regarding the protein composition of the plasma, loss of specificity and selectivity (106), specific and standardized in vitro assays need to be developed and validated. When using biomaterials like plasma or serum, standardized batches are required.

Liposomal formulations are known to cause (pseudo)allergic infusion reactions, by binding to proteins of the complement system (27,91,92). However, no complement-binding tests have been included in the guidance. Meanwhile, the field has developed specific assays for in vitro analysis of formation of complement factors. By using standardized human serum suited for in vitro testing of complement activation (serum that has been characterized for human serum complement factors), these tests can be standardized and used for safety testing regarding complement activation in the quality control of each batch. These complement activation tests are applicable on every liposomal drug formulation as an accurate and straightforward in vitro characterization assay, and therefore should be implemented in the guidance. In addition, FACS determination of aggregates in liposomal formulations could also be used to qualify liposomal homogeneity, and could be used to predict for complement activation already during physicochemical characterization of the liposomal drug product (83).

Regulation of liposomal drug products using a general guidance document

In summary, three levels of liposomal characterization assays are needed to regulate liposomal drug formulations. First, there are the general guidelines regarding drug quality,

efficacy and safety, as developed by ICH and established in respective pharmacopoeias (United States Pharmacopoeia, European Pharmacopoeia and Japanese Pharmacopoeia). These guidelines deal with product specific characteristics, like sterility, pH and osmolality for parenteral products, or hardness and friability of tablets. In addition, tests regarding quality and identity of the API and excipients are given. Second, a more specific guidance regarding liposomal drug products is required, that includes detailed evaluation of liposomal physicochemical properties as discussed above. The draft guidance as developed by the FDA is a good starting point which, however, needs to be updated with test items as suggested in this article. Third, highly product-specific characteristics of features should be tested on a case-by-case basis, to assure the guidance document will remain structured, uncomplicated and widely applicable. During development of a new liposomal formulation, specific tools and methods for the analysis of its unique product characteristics to measure product quality and batch-to-batch variations have to be developed. This critical information regarding unique properties and assessment tools and methods should be shared with regulators upon application for a new drug formulation, and should be used when they are considered to offer additional assurance of quality (57,107). These test items could be considered to be taken up in the guidance if they appear to be applicable for a subgroup of liposomal formulations (e.g. PEGylated liposomes), mentioning the subgroup of liposomes they apply for. This would result in a general, straightforward, and up to date regulatory document on the quality control of liposomes and would improve and simplify regulation of liposomal drug products worldwide.

Conclusion

Nanomedicines form a very diverse group of advanced therapeutics. Therefore, an overall guidance document on the regulation of all nanomedicines considered as one group will not cover all aspects regarding characterization, safety and toxicity evaluation of all these different subgroups of formulations. By dividing the group of nanomedicines into selected subclasses, more specific guidelines can be developed, as the FDA did for liposomal drug products. This draft guidance appears a useful document for the regulation of liposomal drug products, in addition to the existing general guidelines for drug products, and could be adopted by other authorities. However, because liposomal drug products are an ever expanding and evolving group of nanomedicines, the guidance needs continuous update and input from the scientific, industrial and clinical field around it. Such may likely also be true for other subclasses of nanomedicines for which specific guidelines will be developed in the future. Besides this, a case-by-case approach will eventually always be necessary to a certain extent, and this requires the authorities and their experts in the review board to keep pace with the scientific developments and innovations with regard to future nanomedicinal products.

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Summary & Future perspectives

Summary

Liposomes have proven to be well tolerated drug delivery vehicles that offer the possibility of targeted drug delivery for a wide range of therapeutic agents. The physicochemical properties of liposomes can be tailored to optimize drug delivery and retention at the target site, thus enhancing their therapeutic efficacy, and to prevent toxicity to non-target tissues. Furthermore, liposomes can offer a solution in case of formulation problems of the active compound (e.g. to overcome low solubility) or to prevent metabolic degradation. In this thesis, pharmaceutical, preclinical and clinical aspects of the development of liposomal glucocorticoids are investigated and discussed.

In **Chapter 1**, an overview of liposomal drug formulations studied for the treatment of rheumatoid arthritis (RA) in the preclinical as well as clinical setting, addressing both local as well as systemic administration, is given. It covers the use of liposomes as carriers for existing antirheumatic drugs and new experimental agents for the treatment of RA. Optimal liposomal properties depend on the administration route: large-sized liposomes show good retention upon local injection, while small-sized liposomes are better suited to achieve passive targeting upon intravenous infusion. PEGylation reduces the uptake of the liposomes by liver and spleen, and increases the circulation time (so-called 'long-circulating liposomes', LCL), resulting in increased localization at the inflamed sites. The phenomenon of 'passive targeting' to pathological sites can be attributed to locally enhanced permeability of the vascular endothelium, allowing long-circulating small-sized PEG-liposomes to extravasate and accumulate in the extravascular tissue, known as the 'enhanced permeability and retention' (EPR) effect. Additionally, targeting ligands can be attached to the liposomal surfaces to achieve selective delivery of the encapsulated drug to specific target cells in RA, referred to as 'active targeting'.

Glucocorticoids (GCs) have proven to be powerful drugs in the treatment of rheumatic diseases, and are useful both as temporary therapy, until the response to other slower-acting drugs (such as methotrexate) is achieved, and as chronic therapy in severe RA that is not well controlled with the standard of care treatment. However, the use of GCs is hampered by unfavorable pharmacokinetic behavior (high clearance rate and a large volume of distribution). This necessitates high and frequent dosing to maintain therapeutic levels at sites of inflammation, which increases the risk for severe adverse effects. To improve their therapeutic index, GCs can be encapsulated in long-circulating liposomes. In search for an optimal liposomal GC formulation for RA treatment we compared the influence of the potency and clearance rate of the encapsulated GCs on the therapeutic activity and the occurrence of adverse effects (**Chapter 2.1**). It was shown that the use of a GC of higher potency (dexamethasone phosphate (DXP)), increases the therapeutic activity of liposomal GC. However, an increase of the systemic levels of free GC after intravenous administration of liposomal GC was observed as well. An inverse relationship was shown between the

systemic free GC levels and the clearance rate of the GC used. As the systemic levels of free GC can cause systemic side effects, this study stresses the importance of a high clearance rate of the encapsulated GC for achieving minimal free drug levels in the circulation. Therefore high-clearance GCs, like budesonide phosphate (BUP), may be good candidates to optimize the therapeutic index of liposomal GCs in the systemic treatment of RA.

Despite their benefits, PEGylated liposomes are also known to cause hypersensitivity reactions in many patients (numbers of up to 30% have been reported). It is thought that the PEGylation profile of the liposome is responsible for activation of the complement system, part of the innate immune system, and leads to hypersensitivity reactions. Most of these reactions are transient and mild, but in some cases hospitalization is needed. In **Chapter 2.2** it was investigated whether variations in the 'PEGylation-profile' of the liposomal surface affect activation of the complement system. Changes in PEG-chain length, PEG concentration at the liposomal surface and liposomal size did however not significantly reduce the activation of the complement system. All formulations tested caused mild activation of the complement system in vitro, however, occasional hypersensitivity reactions in vivo cannot be excluded. One particular formulation, however, wherein the PEG is anchored to cholesterol, turned out to be an extremely strong activator of the complement cascade. Further study of this phenomenon might be useful to elucidate the mechanism behind complement activation by liposomal formulations in general.

Liposomal dispersions have to be stored at 2-8°C to minimize oxidation and hydrolysis of the phospholipids in the liposomal membranes. Dry products generally show higher stability. In an attempt to improve the stability of the liposomal formulation, it was investigated whether hydroxypropyl-β-cyclodextrin (HPβCD) is able to stabilize the liposomal membranes during drying of PEGylated liposomes, as compared to the disaccharides trehalose and sucrose (**Chapter 2.3**). HPβCD possesses a unique cyclic oligosaccharide structure, that is regarded safe for use in different routes of administration, including pulmonary delivery and parenteral administration. In this study, PEGylated liposomes loaded with prednisolone phosphate (PLP) were dried by spray-drying or freeze-drying. The dried powders were tested on their residual moisture content, glass transition temperature and amorphous character. Upon reconstitution the mean liposomal size, size distribution and drug retention were determined and the results were compared to the characteristics of the liposomal dispersion before drying. Addition of HPβCD as a lyoprotectant resulted in stabilization of the liposomal membranes during the drying process in both spray-drying and freeze-drying. The unique structure of HPβCD enables efficient replacement of the water molecules at the liposomal surface during drying of the formulation, thereby protecting the liposomal membranes from damage and keeping its structure intact. Additionally, its relatively high T_g' protects the membranes against damage by ice crystal formation during the freezing phase of the lyophilization cycle, while during spray-drying its relatively high T_g prevents the drying powder from collapse. Upon

reconstitution the resulting liposomes showed similar characteristics to their unprocessed counterparts. Further stability studies are needed to test whether the liposomal formulation indeed has an improved stability upon storage, and can probably be stored even at room temperature.

In **Chapter 3.1** the efficacy and safety of administration of GC in PEGylated liposomes was assessed in a mouse model of experimental arthritis. Liposomal BUP appeared to be more effective at lower dosages than liposomal prednisolone (PLP). Additionally, the suppression of the hormone regulation by the hypothalamic-pituitary-adrenal (HPA) axis, as determined by measuring plasma corticosterone levels, showed faster recovery after administration of liposomal BUP, suggesting less systemic activity compared to liposomal PLP.

While liposomal GCs have proven to be safe in preclinical studies, **Chapter 3.2** presents the first study with targeted delivery of liposomal GC (i.e. PLP) in patients with active RA. The liposomal formulation induces a faster therapeutic onset of the therapy and an improved response to GC, as compared to an equipotent non-liposomal depot injection of methylprednisolone. Intravenous injection of liposomal GCs appears to be an effective strategy to reach all inflamed areas with only a single infusion and as such also provides an attractive alternative to local administration of GCs in multiple inflamed joints. Liposomal GC appeared to be a safe and a novel way to effectively intervene in RA with clear advantages over current RA standard of care based on oral and systemic administration of GCs.

The in vivo performance of a liposomal formulation is critically dependent on its physicochemical characteristics. Typically, small changes in one of these characteristics can have a huge impact on the in vivo behavior of the administered liposomes. Therefore, the characterization of the liposomal formulation is of major importance. In **Chapter 4**, liposomal drug product quality in relation to e.g. excipients used, the manufacturing process applied and the methods of analysis available are reviewed. Moreover, the current regulatory framework regarding (investigational) medicinal products and its implications for liposomal products are discussed. In this discussion, the only specific (draft) guidance document thus far on liposomal drug product quality, as was developed by the FDA in 2002 is taken into account and recommendations and additions for more specific guidance are given. We conclude this draft guidance is a useful document for the regulation of liposomal drug products, in addition to the existing general guidelines for drug products, however, the draft document is eligible for an update to the current state of the art in liposomal physicochemical characterization techniques. Additional testing regarding the product specific characteristics that are not covered by the general document should be defined on a product-by-product-basis. The updated guidance could be used in addition to existing regulatory guidelines, and would improve and simplify regulation of liposomal drug products worldwide.

Future perspectives

Since their first discovery, approximately 45 years ago, the field of liposome research has expanded considerably, and nowadays, liposomes offer a safe, versatile and mature drug delivery platform technology for application in the treatment of a large variety of diseases (1).

Liposomal drug development: administration routes

Over the years liposomes have proven to be well tolerated carrier vehicles, as most liposomes consist of (semi)natural, biodegradable lipids (2). The majority of liposomal medicinal products have been developed as injectables for local or intravenous administration (reviewed for RA in **Chapter 1**), but other, non-parenteral routes of administration have also been explored. Since the lungs provide a large, adsorptive area with a thin, easy permeable mucosal layer and an extensive vasculature network, pulmonary delivery is a very attractive, non-invasive administration route for both local and systemic delivery of drugs. For local delivery, however, rapid drug adsorption from the alveolar epithelium induces the need of frequent dosing, which often is associated with systemic side effects. The use of liposomes in aerosols and dry powders for inhalation results in controlled release of the drug to the lung and a longer drug exposure time, increasing the efficacy of the drug while systemic side effects could be minimized. In contrary, this rapid absorption is an advantage for systemic delivery of drugs by inhalation. Here, liposomes can be used to protect the encapsulated compound against the stress and shear forces caused in generation of the aerosol, to protect the lungs against irritation caused by the compound, and/or to induce controlled release of the compound (3). Despite the advantages, pulmonary delivery of drugs (for both local and systemic delivery) is hampered by the fact that the dosing accuracy is inferior as compared to for instance intravenous administration, since the dose that actually reaches the lungs depends a.o. on the inhalation technique of the patient (3).

Although liposomes are not very stable in the gastro-intestinal tract, liposomes have also been studied to improve the oral delivery of mainly protein and peptide drugs. Liposomes have been found to improve the systemic absorption of a variety of poorly soluble and/or absorbable compounds after oral administration (4). Furthermore, some liposomal formulations can protect the encapsulated drug from enzymatic degradation in the GI-tract (5).

Liposomal formulations have been investigated and applied in the field of topical drug therapy. Dermally applied formulations (lotions or creams) containing liposomes that are able to penetrate the stratum corneum have been developed. Other liposomal formulations have proven successful in the topical delivery of complex and vulnerable drug molecules, such as proteins, peptides, vaccines and pDNA (6).

Liposomal drug delivery: targeting and drug release mechanisms

Liposomes offer the possibility of targeted drug delivery for a wide range of therapeutic agents (2). The vesicular structure of the liposomes enable encapsulation of hydrophilic compounds in the aqueous core as well as hydrophobic compounds in the lipophilic bilayer. The physicochemical properties of the liposomal membrane can be changed to optimize drug delivery across biological barriers (even the blood brain barrier (7)) and the retention at the target site, and to prevent premature (enzymatic) degradation of the encapsulated compound (1,5). In theory, an optimal liposomal drug delivery system 1) selectively accumulates at the pathological target tissue; 2) acquires access to the target cells; 3) unloads its content in a controlled manner only at this target site; 4) avoids healthy, non-target tissues. The liposomal surface can be modified by introducing surface attached ligands or target molecules that are selectively recognized by the target organ and/or target cells (8). Another interesting approach for targeted drug delivery has been the use of liposomes containing ferromagnetic material, loaded inside the liposome or located at the membrane surface. By applying a magnetic field, the liposomes once injected can be guided to the target site in vivo (8), referred to as 'physical targeting'.

Subsequent to the interaction with the target organ and/or target cells, the liposomes have to release their content in a controlled way. To this end, molecules that are sensitive to pathological changes in the environmental conditions can be attached to the liposomal membrane, e.g. pH- and/or temperature sensitive polymers. Besides endogenous pathophysiological triggers, the release may also be induced by external triggers, such as local hyperthermia generated with electromagnetic radiation or ultrasound in the target area (9,10). For magnetic liposomes, alternating magnetic fields have shown to trigger the release of the encapsulated compound(11). Recently, an article about so-called 'fliposomes' was published, describing the use of amphiphiles that undergo a pH sensitive conformational flip and thereby causing the compounds to be rapidly released, especially in acidic environment. These 'fliposomes' can serve as viable drug delivery systems that promptly release their encapsulated content upon small variations of pH in the environment, as for instance in tumor tissue (12). Combination of properties like these may further improve the level of control and make it even more selective. Their apparent compatibility with the newest generation of environment-responsive modalities and drug release triggers is likely to keep liposomes at the forefront of selective and controlled drug delivery technologies for some time.

The circulation time of liposomes can be increased by coating liposomal surface with a hydrophilic layer of oligosaccharides, glycoproteins, polysaccharides and synthetic polymers in order to render liposomes "invisible" for scavenger cells of the mononuclear phagocyte system. Long-circulating liposomes with lipid-anchored PEG on the surface remains the golden standard (13). A disadvantage of PEG, however, is that it is not biodegradable, and

that it might cause hypersensitivity reactions in sensitive patients. Many alternatives for PEG have been explored, like poly(amino acid)s, poly(glycerol), poly(2-oxazoline)s, poly(acrylamide) and poly(vinylpyrrolidone)s. However, none of these alternatives has been studied extensively concerning their biocompatibility, degradation and excretion profiles. Additionally, all of them caused complement activated hypersensitivity reactions, similar to PEG (13). The only formulation that was shown not to activate the complement system was a PEG-free DSPC:CHOL (2:1) liposome formulation with a particle size smaller than 70 nm. These liposomes also showed a prolonged circulation behavior and targeting to inflamed tissues (14). We showed in **Chapter 2.2** that by changing the PEG-chain length, PEG concentration at the liposomal surface and liposomal size, complement activation is not significantly reduced. All tested PEGylated liposomes showed activation of the complement system in normal human serum, however, the level of activation is that low that it likely does not represent a major risk for hypersensitivity reactions. However, one particular formulation, wherein the PEG is anchored to cholesterol, turned out to be an extremely strong activator of the complement cascade. It can be hypothesized that the hydrodynamic forces on the hydrophilic PEG chain results in a tendency to extract the cholesterol anchor out of the lipid membrane, exposing the antibody-binding portion of cholesterol to natural anti-cholesterol antibodies, thus introducing a new trigger for complement activation by these vesicles. As it has been shown in previous studies, anti-cholesterol antibodies are very strong complement activators (15,16). Obviously, future studies will have to test the validity of this hypothesis and might be useful to elucidate of the mechanism behind complement activation induced by liposomes in general.

Manufacturing and quality control

The manufacturing of liposomes involves complex procedures. A range of techniques and methodologies have evolved over the past decades, both at large clinical scales as well as at smaller laboratory scales (1). Liposome manufacturing methods often require a degree of delicacy so as to leave sensitive and vulnerable compounds intact (e.g. therapeutic proteins, pDNA). This can be conflicting with the high temperatures needed to hydrate the lipids and downsize the liposomal dispersion and the mechanical stress and shear caused by the different sizing methods (e.g. sonication, high shear homogenization or high pressure techniques).

Another disadvantage relates to the low entrapment efficiency obtained with most liposome manufacturing methods. For some structures it is possible to load the liposomes after preparation, as for instance by reversed-phase evaporation, injection, freeze-thawing or by a remote loading process using a transmembrane gradient. Especially pH gradients have proven attractive, because of the applicability to a wide variety of amphiphillic drugs or pro-drugs (17,18).

Large scale production of liposomal formulations can pose a challenge. Most of the laboratory-scale methods cannot be easily scaled up for production of larger batch sizes. Also, scale up brings about its own challenges, such as controlling size and size distribution and the sufficient removal of (toxic) organic solvents and free, unencapsulated drug (18). Additionally, no multi-applicable and fully acceptable sterilization technique is available, since sterile filtration does not remove encapsulated viruses and endotoxins, heat sterilization might destroy the liposomal structure, and gamma sterilization might cause degradation of the active ingredient, cholesterol and the phospholipids in the liposomal membrane (18,19).

Liposomes consist of phospholipids that have to be chemically synthesized or semi-synthesized. With the use of animal derived and semi-synthetic lipids products viral or protein contaminations, such as BSE may occur. In the case of synthesized lipids, in process controls (IPCs) should be executed on the intermediate products, to exclude the formation of possibly toxic side products. Since small changes in quality and purity of lipid excipients can have a major effect on the quality and safety of the final liposomal drug formulation, the lipid excipients have to be meticulously characterized (20). This makes lipid excipients quite expensive while it also increases the risk of lipid batch rejection during quality control.

Liposomal phospholipids, especially when dispersed in water, can undergo oxidation or hydrolysis (21). This could induce fusion of liposomes, leakage of the enclosed drug compound, and structural transformations of the liposomes, influencing both physical and chemical stability of the liposomes, eventually changing their performance. Dry products generally show higher chemical and physical stability and a longer shelf life. In **Chapter 2.3** it was shown that HPβCD is a suitable carbohydrate to protect the delicate liposomal membrane during the drying process. Its physicochemical properties, in this case mainly its high T_g' and a high T_g and possibly also its unique molecular structure with a large number of hydrogen donors and acceptors, combined with its safety in numerous administration routes, makes it an interesting protectant for further research on the development of a dry liposomal formulation.

As mentioned before, the group of liposomal products on the market and under development is continuously becoming more diverse. This brings out the need for more specific measurement tools and standardized test methods to assess the quality of these new liposomal products, and to carefully characterize the liposomal properties, especially the properties which are critical for the safety, efficacy and performance of the liposomes. Manufacturers and regulatory authorities together need to fill in the gaps in scientific knowledge and analytical possibilities to ensure the quality of liposomal formulations, as is concluded in **Chapter 4**.

Liposomal glucocorticoids: do they deserve a place in the treatment of rheumatoid arthritis?

Liposomes have proven to be very effective in improving the pharmacokinetics and biodistribution of selected encapsulated drugs, thereby enhancing the therapeutic index. Liposomal products have already acquired a place in the treatment protocols of certain cancers, notable examples being Caelyx®/Doxil® and Myocet® (both liposomal doxorubicin formulations). Despite the advantage of an increased therapeutic index, Caelyx and Myocet show relatively modest prescription numbers (information from our own Institute) as compared to non-liposomal doxorubicin products. Apparently, after market introduction additional hurdles are in the way, likely a result of high market prices of liposomal products, which are at least several times higher than their non-liposomal equivalent (22).

Non-liposomal GCs have been used in the treatment of RA for a long time. In 2010, the European League Against Rheumatism (EULAR) presented a proposal for an updated treatment algorithm for RA, in which the use of GC is advised to be kept to a minimum because of toxicity concerns (23). Liposomal GC are selectively targeted to the inflamed joints, increasing the local efficacy of the drugs meanwhile reducing the systemic exposure and toxicity (24). Therefore, the question now is whether the increased therapeutic index of GCs achieved with this new product really can be translated to a clinical advantage and eventually allows GCs to regain their place in the management of RA.

In answer to this question, **Chapter 3.2** presents the first clinical study on administration of liposomal GCs into patients with active RA. The liposomal prednisolone phosphate formulation induced a faster therapeutic onset of the therapy and an improved response to GC, as compared to a depot injection of (non-liposomal) methylprednisolone. Indeed, the study gives a first indication of the potential therapeutic value of the liposomal product: a safe and novel way to effectively intervene in RA via a single infusion. If liposomal GC turns out to have disease modifying properties (which the latest preclinical studies with liposomal GC seem to point to (25-27)), the place of liposomal GC in the future RA treatment algorithm might become more pronounced. However, larger studies are needed to further investigate the relative benefit, safety and efficacy of liposomal GC formulations.

Additionally, preclinical studies in **Chapter 2.1 and 3.1** have shown that the use of budesonide phosphate instead of prednisolone phosphate can further increase the therapeutic index of liposomal GCs. Liposomal budesonide was as effective as prednisolone at only one tenth of the dose, while less systemic side effects were seen as a result of its higher clearance rate. While budesonide is currently only used in topical treatment modalities, these results show that it is an excellent candidate for future research of (liposomal) GC therapy in RA.

To conclude, the work presented in this thesis aims to show that liposomes can effectively be used as intravenous targeted drug delivery vehicles for the treatment of RA. Selective delivery of a drug to the inflamed joints can improve the efficacy, while the systemic exposure of the drug is reduced. As a result of the long-circulating and targeting properties of PEGylated liposomes, the dose level and dosing frequency can likely be reduced dramatically. There may be possibilities to even further improve the therapeutic index by selecting GCs with a more suitable pharmacological profile as well as by continued research for options to modify the lipid bilayer in order to reduce the occurrence of hypersensitivity reactions. Also, the work presented in this thesis shows that the manufacturing process, characterization procedure and stability of the final product leave some room for improvement. Clearly the pharmaceutical development of liposomal GC products is not yet finished and, while undertaking our efforts, we continue to expand our knowledge and expertise with regard to this field of targeted drug delivery.

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Appendices

Samenvatting

Dankwoord

Curriculum Vitae

List of Publications

Samenvatting

Liposomen zijn minuscule vetblaasjes ter grootte van een duizendste tot een tienduizendste millimeter waarin geneeskrachtige stoffen kunnen worden ingesloten. Ze bestaan uit een waterige kern omhuld door een zogenaamde fosfolipiden bilaag, en zijn daarmee verwant aan biologische structuren zoals bijvoorbeeld de celmembraan. Sinds hun ontdekking, zo'n 45 jaar geleden, hebben liposomen bewezen effectieve, goed verdraagbare geneesmiddel-transport-deeltjes te zijn. Zij bieden de mogelijkheid een breed scala aan geneeskrachtige stoffen op selectieve plaatsen in het lichaam af te leveren ('targeted drug delivery' of gestuurd geneesmiddeltransport). Door de fysisch-chemische eigenschappen van de liposomen te variëren kan de selectiviteit ten aanzien van de afleverlocatie, de manier van aflevering en verblijfsduur van het in het liposoom ingesloten geneesmiddel worden geoptimaliseerd. Hierdoor kan de therapeutische werking van het ingesloten geneesmiddel worden verbeterd. Tevens wordt voorkomen dat het geneesmiddel in andere organen en/of weefsels dan het doelorgaan terecht komt en zo bijwerkingen kan veroorzaken. Ook kunnen liposomen gebruikt worden om geneesmiddelen die slecht wateroplosbaar zijn toch in oplossing te krijgen. Ten slotte kunnen liposomen bescherming bieden aan stoffen die anders al afgebroken worden in het lichaam alvorens ze de plek van werking bereikt hebben.

Dit proefschrift richt zich op de farmaceutische optimalisatie van de liposomale formulering, alsmede op de toediening van liposomale glucocorticoïden (een groep geneesmiddelen die vaak gebruikt wordt bij de behandeling van reumatoïde artritis) aan proefdieren en mensen.

Hoofdstuk 1 van dit proefschrift geeft een literatuuroverzicht van liposomale formuleringen die getest zijn voor de behandeling van reumatoïde artritis. Reumatoïde artritis, kortweg reuma, is een chronische inflammatoire aandoening die zich manifesteert in de vorm van ontstoken gewrichten. Deze ontstekingen kunnen op den duur resulteren in onherstelbare gewrichtsschade. Voor de behandeling van reumatoïde artritis met liposomen kunnen de geneesmiddelen toegediend worden in de ontstoken gewrichten (lokale toediening), maar ook worden geïnjecteerd in de bloedbaan (intraveneuze toediening). De optimale eigenschappen van de liposomen zijn verschillend per toedieningsroute. Zo is de verblijfsduur van liposomen in het gewricht na lokale toediening langer wanneer de liposomen groter zijn, terwijl de liposomen juist klein moeten zijn om de plek van werking te bereiken na intraveneuze toediening. Daarnaast kan het oppervlak van de intraveneus toegediende liposomen gemodificeerd worden met polymeren, zoals bijvoorbeeld polyethyleenglycol (PEG), waardoor ze minder snel geïnactiveerd worden door de cellen van het immuunsysteem in de lever en de milt. Hierdoor krijgen deze liposomen langer de tijd hun doel te bereiken. Deze lang circulerende liposomen kunnen uit de bloedbaan treden in de ontstoken gewrichten, waar het endotheel (de wand) van de bloedvaten poreus is. De liposomen hopen zich op in het ontstoken gewricht, waar ze door de cellen van het

immuunsysteem (zogenaamde macrofagen) worden opgenomen. Dit zorgt voor vrijkomen van het ingesloten geneesmiddel, precies op de plek waar het zijn werking uit moet voeren. Dit noemen we passief gestuurd geneesmiddeltransport. Dit gestuurde geneesmiddeltransport kan nog verder geoptimaliseerd worden door specifieke biologische stoffen zoals eiwitten en suikerverbindingen (die specifieke interacties aangaan met de zieke cellen op de aangedane plek) op het oppervlak van de liposoom te bevestigen. In dit geval spreken we van actief gestuurd geneesmiddeltransport. In het hoofdstuk worden de verschillende vormen van toediening en transport van liposomen besproken voor zowel reeds bestaande geneeskrachtige stoffen in de behandeling van reuma, als voor nieuwe stoffen die werkzaam zouden kunnen zijn tegen reuma.

Glucocorticoïden zijn ontstekingsremmers die in de klinische praktijk gebruikt worden om ernstige uitbarstingen van ontstekingen bij reumatoïde artritis te onderdrukken. Deze middelen kennen echter forse bijwerkingen omdat ze tevens terecht komen in organen waar ze ongewenst zijn. Daarnaast worden glucocorticoïden snel door het lichaam afgebroken en uitgescheiden, waardoor de frequentie van toedienen en de toegediende doseringen hoog zijn. Dit vergroot de kans op ernstige bijwerkingen, en daarom houdt men glucocorticoïden vaak achter de hand, als 'laatste redmiddel'. Door glucocorticoïden in te sluiten in lang circulerende liposomen wordt hun 'therapeutische index' (de balans tussen effectiviteit en bijwerkingen) verbeterd, zoals al eerder is aangetoond in verschillende dierstudies.

In **hoofdstuk 2** wordt de liposomale glucocorticoïd-formulering nader bestudeerd. Allereerst worden in **hoofdstuk 2.1** de optimale eigenschappen van het ingesloten glucocorticoïde onderzocht. Het blijkt vrijwel onmogelijk te voorkomen dat de stof in zijn vrije vorm in de bloedbaan terecht komt. Aangezien deze vrije vorm in de bloedbaan de veroorzaker is van bijwerkingen, laten wij zien dat het meest optimale glucocorticoïde een stof is die zo snel mogelijk wordt afgebroken en afgevoerd ('geklaard') uit de bloedbaan. We laten tevens zien dat budesonide, een glucocorticoïde dat momenteel voornamelijk ter inhalatie gebruikt wordt bij allergieën en luchtwegaandoeningen, een goede kandidaat is als liposomale ontstekingsremmer, omdat de vrije fractie sneller geklaard wordt dan bijvoorbeeld de glucocorticoïden dexamethason en prednisolon.

Vervolgens is onderzocht of de liposomale drager kan worden geoptimaliseerd. Ondanks de voordelen die PEG biedt aan de eigenschappen van het liposoom, kleven er ook enkele nadelen aan het gebruik van PEG. PEG-gecoate liposomen kunnen het complementsysteem, een onderdeel van het immuunsysteem, activeren. Dit leidt tot overgevoeligheidsreacties bij een aantal patiënten (de literatuur meldt aantallen tot 30% van de behandelde patiënten). Meestal zijn deze reacties mild en verdwijnen de symptomen snel wanneer de infusie wordt gestopt, maar ernstigere gevallen zijn ook gerapporteerd. In **hoofdstuk 2.2** is onderzocht of door de PEG-coating op het oppervlak van de liposomen te variëren (in bijvoorbeeld PEG dichtheid en lengte van de PEG-moleculen) de activering van

het complementsysteem kan worden verminderd. Ook de invloed van de grootte van de liposomen en het effect van wel of niet insluiten van prednisolon is onderzocht. Al deze factoren blijken de activering van het complementsysteem echter niet significant te veranderen. In al deze gevallen is de PEG-keten verankerd aan DSPE (1,2-distearoyl-phosphatidylethanolamine, een van de bouwstenen van de fosfolipiden bilaag). Wanneer we de PEG-keten echter verankerden aan cholesterol (een andere bouwsteen uit de fosfolipiden bilaag) blijkt het complementsysteem extreem sterk geactiveerd te worden. Dit is uiteraard ongunstig als nieuwe liposomale formulering, maar deze liposomen kunnen wellicht wel gebruikt worden om het mechanisme achter het activeren van het complementsysteem verder op te helderen.

Gedroogde producten zijn vaak langer houdbaar en minder gevoelig voor ontleding dan producten die vocht bevatten. Dit geldt ook voor liposomale formuleringen. De fosfolipiden bilaag is echter een kwetsbare structuur die beschermd moet worden gedurende het droogproces. Hiervoor worden vaak suikers gebruikt, zoals bijvoorbeeld sucrose (tafelsuiker) en trehalose. In **hoofdstuk 2.3** is getest of hydroxypropyl-β-cyclodextrin (HPβCD, een bijzondere suikerstructuur, bestaande uit 8 suikermoleculen in ringstructuur) in staat is om de fosfolipiden bilaag te beschermen tijdens het droogproces. De twee meest gebruikte technieken om droge formuleringen te ontwikkelen zijn sproeidrogen en vriesdrogen. Bij sproeidrogen wordt de oplossing verneveld tot fijne druppeltjes, waarna deze druppeltjes in de warme lucht drogen tot poederdeeltjes. Bij vriesdrogen wordt de oplossing ingevroren, en door verlaging van de druk gaat het bevroren water over in de gasvorm en 'verdampt' zo uit de formulering. Het grote verschil tussen de technieken is de droogtemperatuur: deze is hoog bij sproeidrogen, maar kan zelfs onder het vriespunt zijn bij vriesdrogen. Na drogen zijn de karakteristieken van de gevormde poeders (residuaal vocht, glas transitie temperatuur, amorf karakter) onderzocht. Vervolgens zijn de poeders weer opgelost en zijn de karakteristieken van deze liposomen vergeleken met dezelfde liposomen van vóór het droogproces (grootte, deeltjesgrootte-verdeling, gehaltes vrij en ingesloten prednisolon). HPβCD blijkt in staat de liposomen te stabiliseren in beide droogprocessen. De liposomen na drogen waren gelijkwaardig aan de liposomen voor drogen. Dit is te wijten aan twee belangrijke eigenschappen van HPβCD: het zorgt voor de vorming van kleine ijskristallen tijdens bevriezen en voorkomt daarmee schade aan de fosfolipiden bilaag door ijskristallen (het heeft een hoge T_g'). Daarnaast gaat het pas bij hoge temperaturen over van amorfe naar kristallijne structuur (hoge T_g), en voorkomt daarmee het 'instorten' van het beschermende netwerk rondom de fosfolipiden bilaag bij hoge temperaturen tijdens het sproeidroogproces.

Hoofdstuk 3 beschrijft het gebruik van liposomale glucocorticoïden bij dieren en mensen. **Hoofdstuk 3.1** laat zien dat liposomaal budesonide in muizenstudies inderdaad minder bijwerkingen veroorzaakt (met name de onderdrukking van de hypothalamus-hypofyse-as, een belangrijk hormonaal reguleringsmechanisme in het lichaam) ten opzichte van dezelfde

dosis prednisolon. Daarnaast is de ontstekingsremmende activiteit van budesonide hoger waardoor een lagere dosering dan liposomaal prednisolon toereikend is om hetzelfde ontstekingsremmende effect te bereiken. Dit laat zien dat budesonide inderdaad potentie heeft om te worden toegevoegd aan het arsenaal van liposomale glucocorticoïden voor de behandeling van reumatoïde artritis.

Hoofdstuk 3.2 beschrijft de eerste studie van met PEG-gecoate, lang circulerende, prednisolon bevattende liposomen bij de mens (in dit geval patiënten met actieve reumatoïde artritis). De liposomale formulering geeft een sneller en beter therapeutisch effect dan een vergelijkbare dosis van een niet-liposomaal depot-preparaat. Intraveneuze toediening van liposomale glucocorticoïden blijkt een effectieve manier te zijn om alle ontstoken gewrichten tegelijkertijd te bereiken met maar één injectie, en is daarom een aantrekkelijk alternatief voor lokale injecties van niet-liposomale glucocorticoïden. Liposomale glucocorticoïden lijken daarmee een veilig en effectief nieuw alternatief bij de behandeling van reumatoïde artritis. Aanvullende, grotere studies zijn nodig om de relatieve voordelen, veiligheid en werkzaamheid van liposomale glucocorticoïden verder te onderzoeken.

Hoe een liposomale formulering zich gedraagt in het menselijk lichaam is afhankelijk van zijn fysisch-chemische eigenschappen. Kleine veranderingen in bijvoorbeeld de grootte of de vorm van het liposoom of de concentratie van de (PEG)-coating kunnen gevolgen hebben voor de effectiviteit of de veiligheid van de formulering. In **hoofdstuk 4** wordt de kwaliteit van de liposomale formulering en de invloed die bijvoorbeeld het bereidingsproces en de gebruikte grondstoffen hebben op deze kwaliteit, beschreven. Tevens worden de richtlijnen en voorschriften die beschikbaar zijn om deze kwaliteit de waarborgen, geëvalueerd. Momenteel is de enig beschikbare documentatie een in 2002 door de FDA uitgebrachte concept richtlijn. De voorschriften beschreven in dit document zijn een goed uitgangspunt om de kwaliteit van liposomale formuleringen te waarborgen, maar moeten worden aangevuld met de huidige kennis en technieken op het gebied van karakterisering van liposomen. Gezien de grote diversiteit in liposomale formuleringen zullen tevens specifieke analyses gedefinieerd moeten worden die gelden voor één of enkele producten. Deze product-specifieke tests zullen niet opgenomen worden in de algemene richtlijn voor liposomale formuleringen. Hierdoor ontstaat een documentatie-structuur op 3 niveaus: 1) Algemene, reeds bestaande richtlijnen en voorschriften om de kwaliteit van geneesmiddelen te waarborgen, 2) Een specifieke richtlijn die geldt voor liposomale formuleringen, en 3) Product-specifieke tests welke niet opgenomen zijn in de richtlijn. Deze structuur zal de borging van de kwaliteit van liposomale formuleringen aanzienlijk versimpelen en verbeteren.

Samenvattend wordt in dit proefschrift aangetoond dat liposomen effectief gebruikt kunnen worden voor gestuurd geneesmiddeltransport van glucocorticoïden bij de behandeling van reumatoïde artritis. De glucocorticoïden hopen zich selectief op in de

ontstoken gewrichten, wat resulteert in hoge concentraties op de plaats van werking, terwijl de concentraties van vrije glucocorticoïden in de bloedbaan (en daarmee het optreden van bijwerkingen) wordt geminimaliseerd. Optimalisatie van de liposomale drager om het optreden van overgevoeligheidsreacties te verminderen vereist verder onderzoek. Tevens is er ruimte om bereidingsproces, analyse en stabiliteit van het eindproduct verder te verbeteren. Dit proefschrift biedt de basis voor verdere ontwikkeling van liposomale glucocorticoïden en 'targeted drug delivery' en daarmee nieuwe behandelingsmogelijkheden voor patiënten met reumatoïde artritis.

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Jolanda

Utrecht 2012

Curriculum Vitae

Jolanda Maria van den Hoven werd geboren op 21 februari 1981 te Amersfoort. In 1999 behaalde zij haar VWO-diploma aan het Meridiaan College 't Hooghe Landt te Amersfoort. Aansluitend werd begonnen aan de studie Farmacie aan de Universiteit Utrecht. In augustus 2003 werd het doctoraal diploma behaald, gevolgd door het apothekersdiploma (met profilering 'geneesmiddelontwikkeling') in Augustus 2005. Direct na haar studie begon zij haar werkzaamheden bij de apotheek van het Slotervaartziekenhuis te Amsterdam, als projectapotheker op het gebied van productie van experimentele cytostatica. In juli 2008 werd in dezelfde instelling, in samenwerking met de Universiteit Utrecht en Enceladus Pharmaceuticals, begonnen met het promotieonderzoek dat is beschreven in dit proefschrift. Het onderzoek werd uitgevoerd onder begeleiding van Prof. Dr. G. Storm, Prof. Dr. J.H. Beijnen, en co-promotoren Dr. B. Nuijen en Dr. J.M. Metselaar. Na haar promotie zal ze werkzaam blijven als projectapotheker productie bij de apotheek van het Slotervaartziekenhuis.

Jolanda Maria van den Hoven was born on February 21st 1981 in Amersfoort, The Netherlands. In 1999 she finished secondary school at the Meridiaan College 't Hooghe Landt in Amersfoort, The Netherlands, she started studying Pharmaceutical Sciences at Utrecht University. In August 2005 she obtained her Pharmacist degree (with specialization 'drug product development'). Immediately after graduation she started working at the Slotervaart Hospital as a pharmacist on the manufacturing of investigational anticancer agents. In July 2008 she started with the PhD project described in this thesis. The research was a collaboration between the Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam; the Department of Pharmaceutics, Utrecht University, Utrecht and Enceladus Pharmaceuticals, Amsterdam, and was supervised by Prof. Dr G. Storm, Prof. Dr. J.H. Beijnen, Dr. B. Nuijen and Dr. J.M. Metselaar. She will continue her work at the pharmacy of the Slotervaart Hospital as a pharmacist specialized on drug product development and manufacturing.

List of publications

van den Hoven JM, Metselaar JM, Storm G, Beijnen JH, Nuijen B. "Regulatory aspects on liposomal formulations. What should be tested and why?" manuscript in preparation

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